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DIFFERENTIAL HORMONAL REGULATION OF RAT ACIDIC EPIDIDYMAL GLYCOPROTEIN IN THE PAROTID GLAND AND THE EPIDIDYMIS

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1994

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Differential Hormonal Regulation of Rat Acidic Epididymal Glycoprotein in the Parotid Gland and the Epididymis

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Beverly E. Naiman
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Abstract

DIFFERENTIAL HORMONAL REGULATION OF ACIDIC EPIDIDYMAL GLYCOPROTEIN IN THE EPIDIDYMIS AND PAROTID GLAND OF THE RAT. Beverly E. Naiman and Nancy J. Charest. Section of Endocrinology, Department of Pediatrics, Yale University, School of Medicine, New Haven, CT.

Acidic Epididymal Glycoprotein (AEG) is a 31,000 molecular weight, well characterized major secretory protein of the rat epididymis whose gene expression is regulated by androgens. Our laboratory has recently discovered that AEG is a major secretory product of the parotid gland as well. The purpose of this study was to investigate the hormonal regulation of gene expression of AEG in the parotid gland and compare it to the regulation of AEG in the epididymis. This was accomplished by various manipulations of hormonal status of rats, and removing the parotid gland and epididymis after treatment. Total RNA was isolated from each tissue and specific levels of AEG mRNA were followed by Northern blot analysis with a radiolabeled AEG cDNA probe.

A developmental profile of AEG mRNA expression in the parotid gland was constructed and compared to that of the epididymis. Significant amounts of AEG mRNA were detectable by 20 days of age, with the most rapid increase occurring between day 25 and 30. Adult levels were reached by day 45 and remained constant through day 90. A very similar developmental pattern was seen in the epididymis. Adult female rats treated with daily injections of testosterone for 24 or 48 hrs had unchanged parotid AEG mRNA levels compared with untreated controls. Androgen resistant males had similar

parotid AEG mRNA levels in the parotid gland compared to their normal male siblings. Castration of adult male rats reduced epididymal AEG mRNA levels but revealed no change in parotid AEG mRNA taken from those same animals. Administration of testosterone two weeks post castration restored epididymal AEG mRNA but had no effect on parotid levels. The effects of the ß-adrenergic agonist isoproterenol (IPR), an agent known to regulate the expression of many parotid proteins, on parotid and epididymal AEG mRNA levels were examined. Parotid AEG mRNA levels were reduced to 12% of controls but epididymal levels were unchanged.

These results indicate that androgens are necessary for epididymal expression of AEG mRNA but not for parotid gland expression. IPR decreases parotid AEG mRNA levels but has no effect on epididymal levels. There is a tissue-specific nature to AEG regulation.

Acknowledgements

I would like to first of all extend my gratitude and appreciation to Dr. Nancy Charest, who has been my advisor, my mentor, and my friend. Without her support, this project would not have been possible. I would also like to thank Dr. Carol Paproski, who was a constant source of day to day knowledge and friendship in the laboratory, Dr. Art Hand, for his invaluable knowledge about the parotid gland and for his time commuting to Yale University from the University of Connecticut, and Mr. Ralph Garcia, the research assistant from across the hall, for his eagerly proffered aid.

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1.0 Introduction

While almost all cells contain the full complement of genomic DNA, individual tissues express only a specific subset of those genes. This selective control of expression is the basic mechanism by which multicellular organisms specialize into tissues with distinct functions. Differentiated cells possess a remarkable capacity for selective expression of different genes. Control of the expression of those genes is mediated by a wide range of compounds and varies from cell to cell interaction, local chemical factors, to hormonal control. When a single gene is expressed by a limited number of tissues, exploring the regulation of the gene in each tissue provides some insights into tissue-specific hormonal regulation of gene expression. This introduction provides a contextual backdrop for understanding the differential hormonal regulation of the gene for acidic epididymal glycoprotein (AEG), a 31,000 molecular weight protein synthesized by both the rat epididymis and the parotid gland. It discusses the mechanisms of hormonal regulation at the cellular and molecular level, provides general background information on both tissues in which the AEG gene is expressed and reviews previous studies of AEG from the literature.

1.1 Hormonal Regulation of Gene Expression

1.1.1 Hormonal Regulation

The ability to respond to extracellular signals is essential for the development and survival of all living organisms. Multicellular organisms have developed the capacity to generate many different internal signals, in the form of hormones and other compounds, and use them for the control and regulation of individual cells. The cellular actions of hormones are coupled to the subcellular functions of the cell. While hormones may exert

The Molecular Mechanism of Steroid Hormone Activation

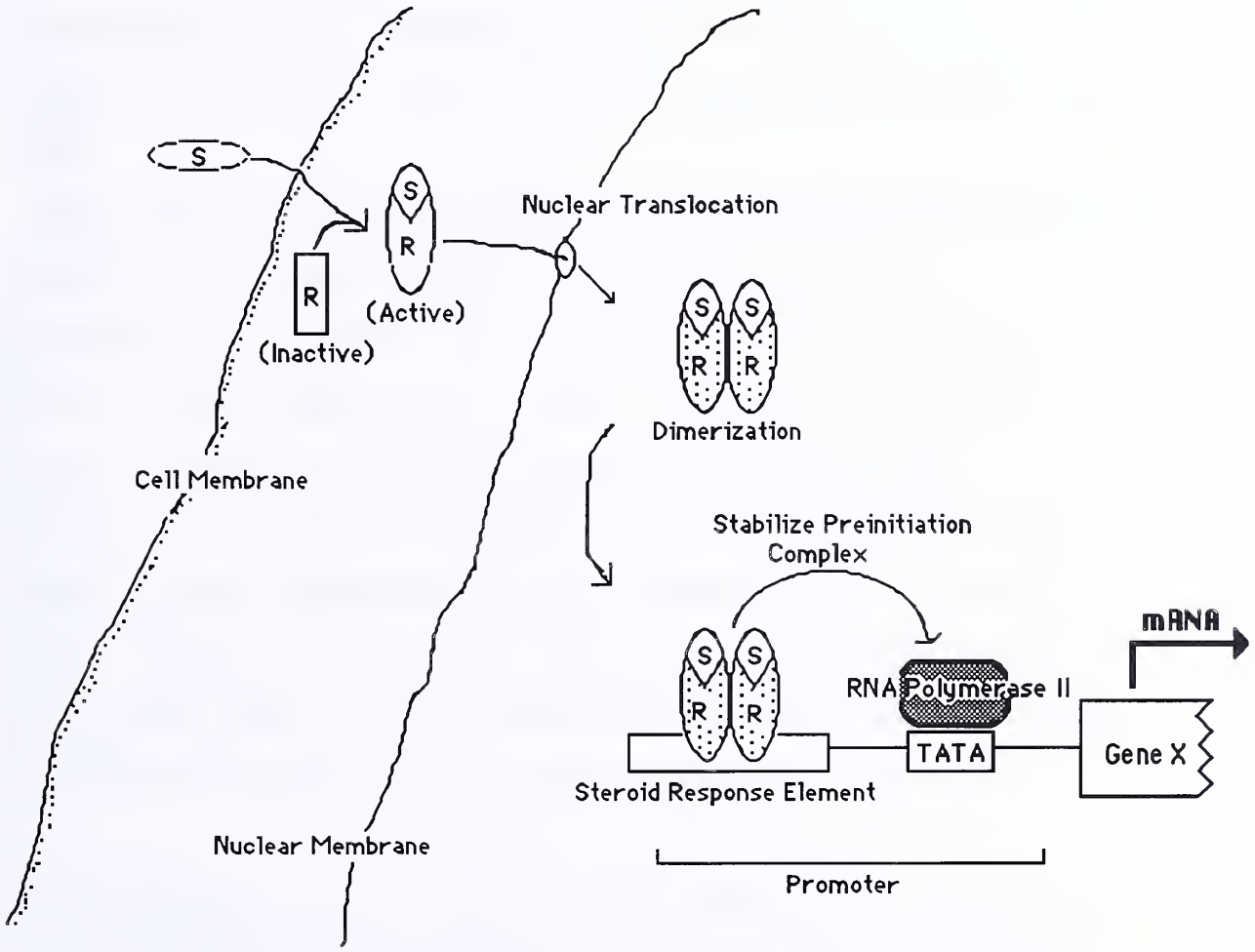


Figure 1: The proposed steps which a steroid hormone ligand takes that lead to activation of gene transcription. (S) steroid hormone ligand, (R) steroid hormone receptor See text for details. [Figure derived from (Clark, 1992)]

referred to as the TATA box, which is required for accurate initiation of transcription at the first nucleotide of the first exon of the structural gene. This binding leads to the production of pre RNA, processing to mRNA, and eventual translation to protein. Other SREs can serve as inhibitors to transcription by several mechanisms, such as when dimer binding physically blocks the promoter site and prevents RNA polymerase from binding (Clark, 1992).

This superfamily of hormone receptors can be divided into two groups: one which includes the glucocorticoid, progesterone, androgen, and mineralocorticoid receptors, and the other which includes the estrogen, thyroid hormone, retinoic acid and vitamin D3 receptors. The primary amino acid sequence of the DNA binding domain displays stricter conservation within these two subgroups; in particular, the amino acids between the two cysteines closest to the C-terminus side on the first "zinc finger" are always Gly-Ser in the first subgroup and always Glu-Gly in the second (Beato, 1989). A better understanding of steroid hormone action can be obtained by examining the steroid effects of androgen on target cells.

1.1.3 Androgen as a model of steroid hormone regulation

Like other steroid hormones, the molecular basis of androgen action appears to be transcriptional regulation (Rundlett, 1990). The androgen testosterone is largely bound to albumin and to steroid-binding globulins in serum. Only the free fraction of testosterone is able to diffuse across the membrane. In tissues which express 5α-reductase, a membrane-bound enzyme associated with both nuclear and microsomal fractions, testosterone may be irreversibly converted to its more active metabolite,

dihydroxytestosterone (DHT) (Robaire, 1988). Androgen receptors have been localized to the cytoplasm in a perinuclear distribution in the absence of hormone ligand, (Simental, 1991) or in the presence of antiandrogens (Kemppainen, 1992). However, other studies have found that even in the absence of steroids there is still a significant nuclear fraction of AR (Jenster, 1991). Testosterone or DHT now binds to the steroid-binding domain encoded by approximately 250 amino acid residues in C-terminus of the androgen receptor (Jenster, 1991) and forms an activated hormone-receptor complex. The receptor contains the amino acid sequence Arg-Lys-Leu-Lys-Lys-Leu-Gly-Asn , starting with amino acid number 628, allowing localization of the AR-DHT complex to the nucleus (Lanford, 1986). In the nucleus, this complex forms a dimer, which is dependent on both the presence of the DHT and an intact NH_2-terminal domain (Wong, 1993). This dimer binds to the androgen responsive element (ARE) sequence of androgen-regulated genes. Unlike some other SREs, such as for glucocorticoid and estrogen, a consensus sequence has not yet been defined. Putative AREs such as AGAACAnnnAGTGCT ("n" represents nucleotide spacing between palindromic sequences) of the prostatic specific antigen, (Riegman, 1991) or GGAACAnnnAGTGCT of human kallikrein (Murtha, 1993) have been described. Once bound to the ARE , the NH_2-terminal of the AR is essential to stimulate the transcription of the gene; deletion of the first 338 amino acids terminates this activity (Simental, 1991).

1.1.4 Surface hormone-receptor regulation

In contrast to steroid hormones, peptide hormone receptors, as well as catecholamine receptors are located on the plasma membrane. The first evidence for this came from the fact that, unlike steroid hormones, antibodies

to peptide hormones can reverse hormone action after it has already begun (Kahn, 1993). Also unlike steroid hormones, the interaction between the hormone and receptor is rapid and reversible; hormone binding to the receptor triggers aggregation and internalization into the cell to allow for rapid turnover and recycling of receptor. Surface receptors ultimately exert their effect through a variety of common pathways to effect cellular transport, membrane channels, enzymatic activation, protein phosphorylation, or gene expression. A general model for peptide hormone or catecholamine regulation involves the interaction of a hormone, a receptor, and an effector which transduces the hormone's signal at the cell surface into a second intracellular message which mediates the hormone's effect inside the cell. There are three general classes of membrane receptors. The first are self-contained systems where ligand-binding directly influences ion channel gating. This receptor type is seen in the nicotinic cholinergic receptor, which operates as a sodium/potassium channel (Changeux, 1989). Another class of membrane bound receptors activates phosphorylation of proteins via tyrosine kinase in response to ligand binding. The best characterized tyrosine-kinase receptor is the insulin receptor (Kasuga, 1982). Finally, ligand binding to surface receptors may be coupled to a G-protein. Second messengers for G-proteins include a wide range of compounds, such as cyclic AMP (cAMP), Ca^{+2}, inositol 1,4,5-trisphosphate (IP_3), etc. These molecules may activate cytoplasmic kinases, which in turn can phosphorylate transcription factors. These modified TFs (PO_4-TFs) form dimers and bind to hormone response elements (HRE), which serve as enhancers. These bind to nuclear transcription factors and alter the rates of gene transcription, which, like steroid hormones, may either be in a positive or negative direction

(Kahn, 1992). Describing in detail a single hormone-receptor interaction serves to illustrate these principals.

1.1.5 ß-adrenergic System as a Model of Surface-Receptor Hormone Regulation

The ß-adrenergic catecholamines act as both a hormone and a neurotransmitter to cells. The catecholamine ligand interacts with the ß-adrenergic receptor to produce its second message. See figure 2. The signal transduction pathway associated with ß-adrenergic receptor activation is linked to a G protein, which is a heterotrimer of three subunits, α, ß, γ. In the absence of ligand, the α subunit is bound to GDP. The binding of the ligand promotes the GTPase activity of the G protein; GTP replaces the GDP on the α subunit. The now activated α subunit dissociates from the ßγ subunits and stimulates the activity of adenylate cyclase, a membrane bound glycoprotein which now converts ATP to cAMP (Casey, 1988). Each new molecule of cAMP can bind to the regulatory subunit of protein kinase A (PKA) and cause the dissociation of the tetrameric kinase into its four catalytic and regulatory subunits. The catalytic (C) subunit then translocates into the nucleus where it is believed to phosphorylate a cAMP response element-binding protein (CREB). Direct evidence that the C subunit of PKA regulates gene transcription comes from experiments showing that *in vitro* , this purified subunit can stimulate transcription of the gene for urokinase-type plasminogen activator (Roesler, 1988). CREB is a 43 kilodalton (kd) transcription factor protein which is a member of a larger superfamily of DNA-binding proteins known as bZIP proteins because they all contain a leucine "zipper", a coiled structure responsible for dimerization (Meyer, 1993). The PKA phosphorylation site resides within the CREB activation domain. Phosphorylation may induce a conformational change resulting in a

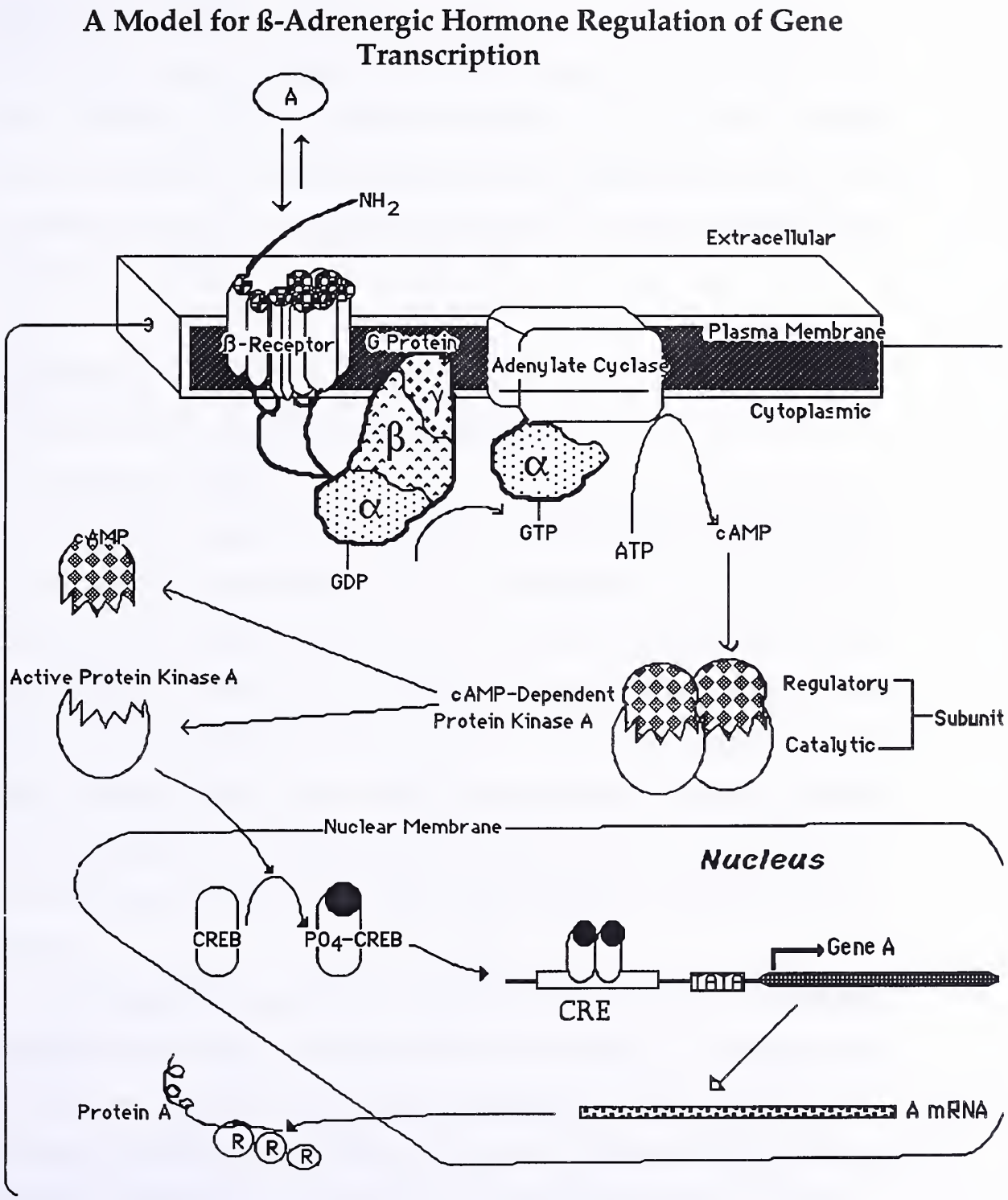


Figure 2: The steps required for a adrenergic peptide hormone to regulate gene transcription. (A) adrenergic hormone ligand, (PO4-CREB) phosphorylated CREB, (CRE) c AMP response element, (R) ribosome See text for other abbreviations and details. [Figure derived from (Kahn, 1992)]

modification of CREB at the site of the transcriptional activation domain. CREB forms a dimer and binds to the cAMP response element (CRE) (Karin, 1991). The CRE is an 8 base palindromic sequence T(G/T)ACGTCA, a highly conserved sequence associated with known CRE-regulated genes, and serves as an enhancer sequence to modulate transcription in a similar manner to the SREs (Roesler, 1988).

1.2 Epididymis

This is one of the tissues which expresses AEG.

1.2.1 Anatomy-Histology

The epididymis consists of a continuous convoluted duct which extends from the upper pole of the testis and ductuli efferentes, to its lower end where it becomes the ductus (vas) deferens. It is encased in the epididymal fat pad. The epididymis is divided into many different segments by connective tissue septa. On a gross anatomical level, three major parts of the epididymis can be defined: the caput (head), corpus (body), and cauda (tail), which are composed of these segments. The segment into which the ductuli efferetes empties is the initial segment, part of the caput, but often considered separately. (See Figure 3)

Histologically, the epididymis is a tube of smooth muscle which ranges from only two or three layers thick in the initial segment to numerous layers more distally, lined by a pseudostratified epithelium. The epithelial cells lining the rat epididymis comprise several types: principal, narrow, clear, and basal cells , which vary in their segment to segment distribution. Narrow cells are found only in the initial segment. Principal cells, the predominant cell type throughout the epididymis, range from a high of 80% of epithelial

Anatomy of the Rat Epididymis

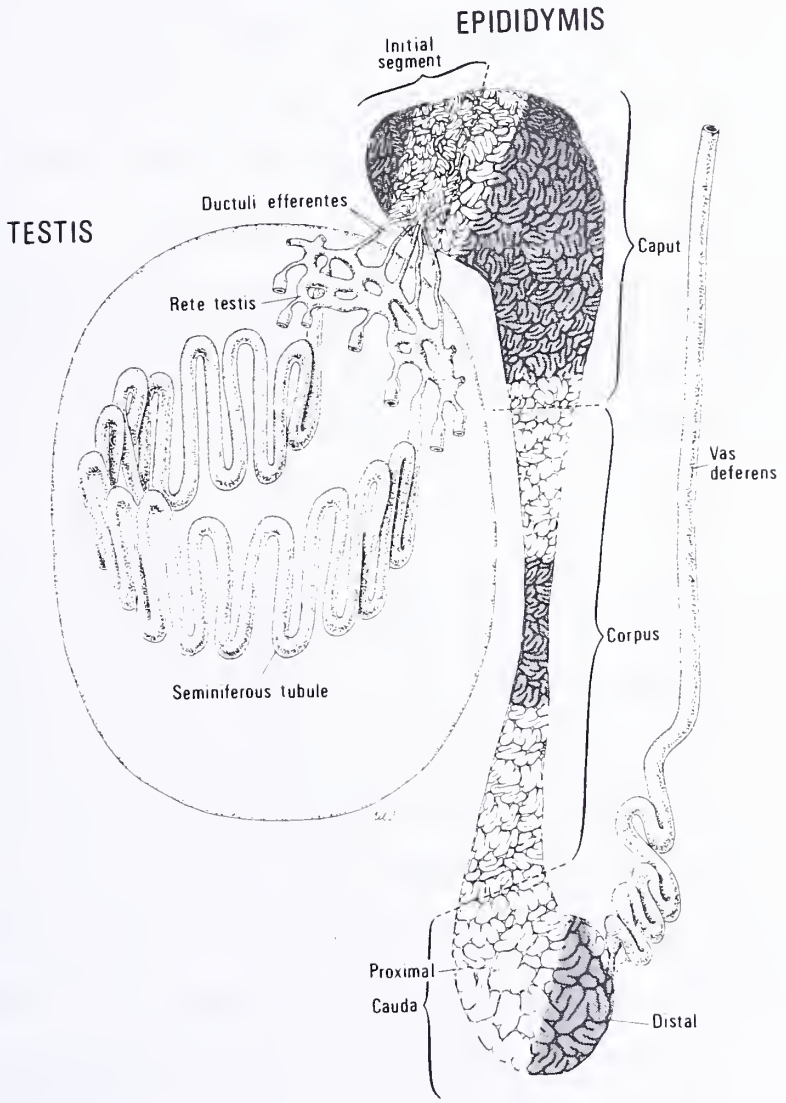


Figure 3: Diagramatic representation of the testes showing a seminiferous tubule and the rete testis, the ductuli efferentes, the epididymis, and vas deferens. The shaded regions indicate areas of the different segments of the epididymis, i.e., the initial segment, caput, corpus, and proximal and distal cauda. (From Robaire, 1988)

cells in the initial segment to 69% in the cauda. The principal cells have tufts of long microvilli, which are thought to be involved in the absorption of the fluid accompanying the sperm. The most striking features of principal cells are large Golgi apparatus and significant amounts of rough and smooth endoplasmic reticulum, which suggests they may also have a significant secretory function. Basal cells are flat cells which lie in contact with the basement membrane, and increase from 12% in the initial segment to 21% in the cauda. Both principal cells and basal cells differentiate from the same precursor, the columnar cell, at around day 28. Clear cells , found mostly in the cauda, are identified by their highly vacuoled apical region and numerous dense granules and are thought to be endocytic (Robaire, 1988).

1.2.2 Innervation

The epididymis is supplied by the middle spermatic and inferior spermatic nerves. These contain both sympathetic and parasympathetic fibers so that throughout the human epididymis there are rich adrenergic and cholinergic plexuses. In the rat, however, there is a only a sparse distribution of nerves in the caput and corpus, but the cauda is well innervated. The fibers of the nerve plexus in the cauda are associated not only with the smooth muscle cells but also appear to approach the basement membrane of the epithelial cells (El-Badawi, 1967).

1.2.3 Embryology and Development

The epididymis is derived from the upper segment of the mesonephric (Wolffian) duct, which is of mesodermal origin. After birth, in the rat, the epididymis goes through a phase of slow growth (weight gain), followed by a rapid growth phase associated with the appearance of sperm in the lumen of

the epididymis (Robaire, 1988). The development of the blood-testis barrier and the seminiferous cord lumen which allows direct passage of sperm and testicular fluid and androgens to the epididymis occurs around 18 days of life in the rat (Setchell, 1988). However, androgens are detectable at significant levels well before this time, suggesting that the bloodstream may be an important prepubertal source (Charest, 1989).

The development of the epididymis requires androgens. One line of evidence for this control comes from investigations using androgen resistant animals, called the testicular feminized or Tfm, originally described in 1970 (Lyon, 1970). In the rat, a single base substitution which changes an arginine to glutamine in the androgen receptor gene results in the expression of an androgen receptor which is unable to bind androgens and has greatly reduced transcriptional activation (Yarbrough, 1990). In the Tfm mouse, androgen receptors are not present. This mutation has been linked to a single base deletion in the N-terminal domain of the androgen receptor (AR) genomic DNA, and is thought to result in the expression of an unstable AR mRNA (Charest, 1991). Genetic males which possess the Tfm mutation are resistant to pharmacological doses of testosterone and lack all Wolffian duct structures, including the epididymis (Orgebin-Crist, 1975). Castration of male fetuses preclude the development of the epididymis, but castrated fetuses treated with crystalized testosterone before sexual differentiation occurs permitts normal epididymal development (Dauzier, 1956). Androgen dependency of the epididymis continues throughout development, as changes in epithelial height, tubular diameter, and staining characteristics occur after castration, and may be restored by administration of exogenous testosterone (Orgebin-Crist, 1975).

1.2.4 Function

A number of different functions have been ascribed to the epididymis. The first is its ability to absorb a large amount of fluid which enters from the testis, resulting in a multifold concentration of spermatozoa, or an increase in the spermatocrit. Another is the storage of sperm, which occurs in the tail of the epididymis in both rats and humans. But perhaps the most important is the secretion of proteins, glycoproteins, and other substances in to the lumen of the epididymis, thought to be involved in sperm maturation (Orgebin-Crist, 1975).

Spermatozoa undergo a series of changes during their transit through the epididymis. This functional maturation results in progressive improvements in their ability to swim, bind to the zona pellucida of the oocyte, and effect fertilization. This functional maturation is not an intrinsic property of the sperm but is androgen dependent and brought about by interaction with epididymal proteins secreted from the epithelium which become associated with the sperm surface (Orgebin-Crist, 1975). On release from testicular Sertoli cells, sperm are not mobile but acquire this capability during transit through the epididymis. Rat spermatozoa from the caput swim in a circular fashion, while those form the cauda will swim in a persistent direction (Bedford, 1975).

There is evidence that changes in sperm membrane proteins play an important role in the acquisition of motility. One characteristic change is the increase in negative charge on the acrosomal membrane which is felt to result from accumulating negatively charged proteins (Orgebin-Crist, 1975). Numerous studies have demonstrated that the mammalian sperm surface undergoes extensive alteration during maturation due to the addition of components secreted by the epididymal epithelium. Sperm recovered from

the corpus of the epididymis have a low fertilizing capacity, but when incubated with epithelium from the cauda, show a significant increase in their ability to bind to the zona pellucida of oocytes (Moore, 1987).

1.2.5 Androgen Regulation

Androgens are known to be the important regulators of epididymal function. Control of the biosynthetic activity of the epididymis is dependent on gonadal androgens. Castration causes a decline in protein synthesis in the epididymis which can be almost completely restored by the administration of testosterone. Total tissue weight declines after castration to 25% of precastration levels in a week, while administration of testosterone promotes an increase in tissue weight. Similar findings have been reported with total mRNA levels (Brooks, 1987).

The regulation of gene expression of many different epididymal proteins by androgens have been well characterized. Several studies have demonstrated that epididymal proteins, such as GP-83 and GP-49, (Liu, 1992) and protein B/C (Brooks, 1987) are regulated by testosterone through castration/replacement experiments. Androgen-regulated synthesis of other epididymal glycoproteins and activation of enzymatic activity have been well described and characterized in the literature (Robaire, 1988).

1.2.6 Androgen Receptors

The androgen receptor has been isolated from cDNA clones isolated from a rat epididymal library and sequenced (Tan, 1988). This sequencing has allowed the development of polyclonal antibodies from synthetic peptides. Immunostaining for androgen receptors in the epididymis localizes to principal epithelial cells as well as to stromal cells. The highest levels of AR

was in the caput epithelial cells, with lower levels towards the corpus and cauda (Sar, 1990). Previous studies have demonstrated a similar receptor distribution pattern using autoradiography following treatment with tritiated DHT (Stumpf, 1976).

1.3 Parotid Gland

This tissue also expresses AEG.

1.3.1 Anatomy-Histology

The major salivary glands consist of three pairs of glands: the parotid, submandibular and sublingual glands. The location of the parotid gland in the rat differs from that of the human. See Figure 4 for details (Hebel, 1986). The salivary glands are classified as compound exocrine tubular-acinar glands characterized by the bunching of numerous secretory units. With the exception of a few mucin producing units, the parotid gland is a serous salivary gland. Each unit consists of an acini which produces secretion and a duct system which regulates the concentration of water and electrolytes and carries the secretion to the oral cavity . The acini are composed of pear-shaped groups of epithelial cells surrounded by a distinct basement membrane. These epithelial cells have ultrastructure consistent with secretory cells: large Golgi bodies, numerous zymogen granules which contain amylase as their primary protein. The intercalated duct is in direct contact with the acinus and is lined with cuboidal epithelial cells whose major secretory product is lysozyme (Martinez-Madrigal, 1989).

1.3.2 Innervation

The parotid gland is innervated by both sympathetic and parasympathetic postganglionic fibers. The sympathetic fibers arise from the superior cervical

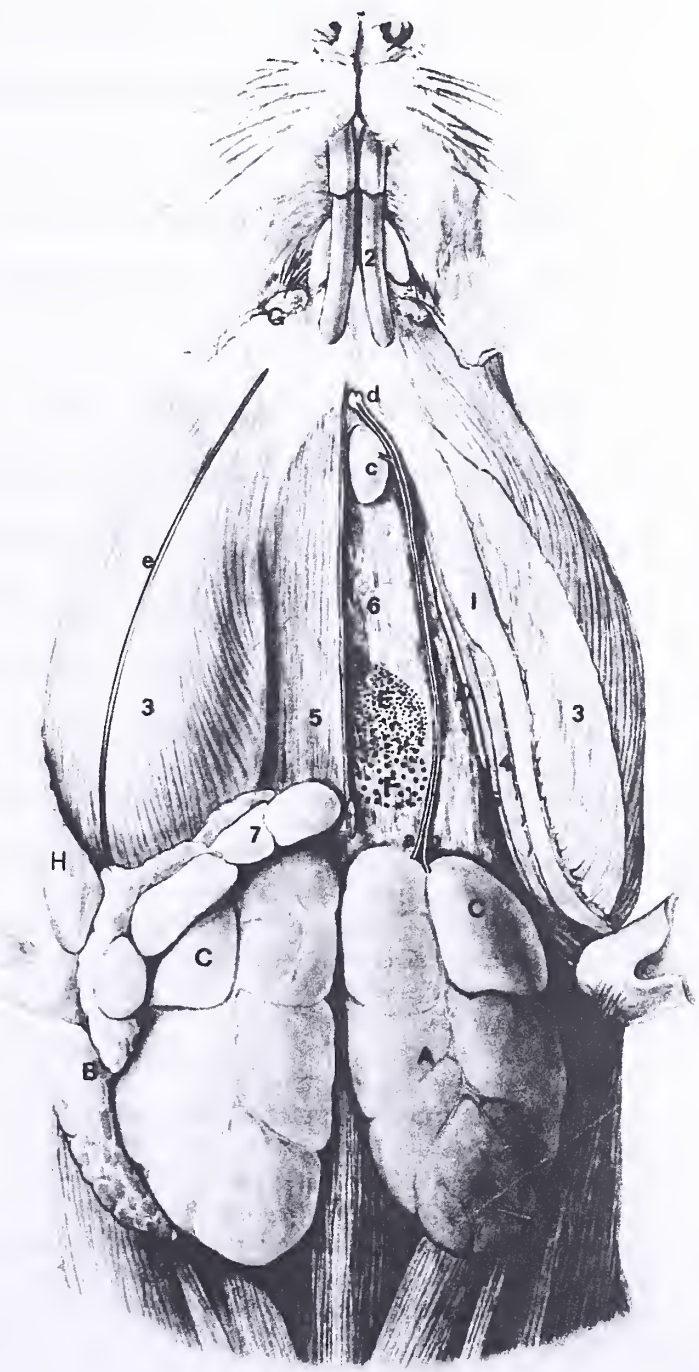
Anatomy of the Ventral Head Region of the Rat Including Parotid Gland

Figure 4: Ventral Head Region (figure taken from Hebel, 1986)

The parotid gland in the rat is attached to the base of the auricle laterally, and runs along the caudal border of the mandible towards the ventral aspect of the larynx. It extends caudally towards the clavicle and rostrally to the extraorbital lacrimal glands. Ventrally, it is covered by the submandibular and sublingual glands. (Hebel, 1986)

The parotid gland (B) is shown only on the left in this figure but it is a bilateral organ.

A Submandibular gland
B Parotid gland
C Sublingual gland, major
D Sublingual gland, minor
E Lingual gland, serous part
F Lingual gland, mucous part
G Sebaceous gland in the oral commissure
H Lacrimal gland, extraorbital
a Mandibular duct
b Sublingual duct
c Enlarged portion of the mandibular duct
d Sublingual caruncula
e Parotid duct
1 Mandible
2 Lower incisors
3 Masseter muscle
4 Pterygoid muscle
5 Mylohyoid muscle
6 Intrinsic muscles of the tongue
7 Lc. mandibulare



ganglion. The parasympathetic innervation to the parotid gland in the rat is supplied by a branch of the auriculotemporal nerve (Schneyer, 1966).

1.3.3 Embryology and Development

The parotid gland derives from the ectoderm as an epithelial bud from the primitive oral epithelium. It is the first of the major salivary glands to appear, around the sixth week in human embryos (Martinez-Madrigal, 1989). This differs from rat embryos, where it is the last of the major salivary glands to appear, around day 14 *in utero*. Lumenization of the parotid ductal system is not complete until day 20 *in utero*., three days later than the other salivary glands. At birth, the terminal clusters of the parotid gland are still relatively undifferentiated; it is the least advanced developmentally of all the salivary glands (Redman, 1970). Although the rat parotid gland is deficient in acini at birth, acinar cells proliferate rapidly during the early post-natal period, reaching a peak in the parotid gland at day 16 after birth (Klein, 1982). Amylase, the major enzyme in the parotid gland, is detectable prenatally, and its activity increases 2-3 days after birth. It is believed that this represents neither a suckling stimulus, nor effect of innervation, but is rather due to the general increase in protein synthesis seen at this time (Lawson, 1970).

1.3.4 Function and Composition of Parotid Saliva

The primary function of the salivary glands is to produce saliva to moisten the mucous membranes of the upper aerodigestive tract. Saliva also helps in the formation and swallowing of the food bolus. In addition, the first stages in digestion of certain compounds, such as starch, is provided by the enzymatic components of saliva.

The rat parotid gland has a high rate of protein synthesis, most of which is of secretory proteins. Amylase represents the principal protein in rat parotid saliva, accounting for almost 30% of the secretory protein and 15-20% of the total protein synthesized by the gland. Anionic and cationic electrophoresis of rat parotid saliva has revealed 21-22 distinct bands of protein, 4 of which are isoenzymes of amylase. The protein composition of parotid secretory granules is identical to that of saliva, and is believed to be secreted via exocytosis (Spearman, 1989).

1.3.5 Regulation

Protein secretion in the rat parotid gland is regulated by a variety of compounds, mainly neurotransmitters, including norepinephrine, acetycholine, substance P, and vasoactive intestinal peptide. These agonists primarily exert their effect through two different signalling pathways. The sympathetic path results in the activation of adenylate cyclase, elevating cAMP and stimulation of cyclic AMP-dependent protein kinase. Parasympathetic stimulation is coupled to the activation of phosphoinositide-specific phospholipase C, resulting in elevation of Ca and protein kinase C activity. It is generally accepted that cyclic AMP-dependent pathway represents the principal method for stimulating protein secretion by exocytosis while the other pathway is more important in controlling water and electrolyte secretion (Spearman, 1989). Acute stimulation of parotid glands with isoproterenol, a ß-adrenergic agonist, results in a rapid depletion of 99% of stored secretory proteins within 2 hrs followed by a more prolonged period of resynthesis, reaching its peak at 6 hrs post injection. While exocytosis has long been known to be mediated through ß-adrenergic receptors, it was not known if the increased rate of protein synthesis

involved the same pathway, since the elevation of cAMP is only transient (Kim, 1989). One recent study investigated the role of ß-adrenergic receptors in regulating gene transcription in the parotid acinar cell using a wide combination of methods. Within 1 hour of ß-adrenergic stimulation, tritiated uridine was incorporated into RNA, showing that induction of gene transcription does occur after the activation of ß-adrenergic receptors in the parotid gland (Woon, 1993).

1.3.6 Chronic stimulation of Parotid Gland

The effects of chronic stimulation over a period of 7 to 10 days of parotid ß-receptors with isoproterenol have been well documented in the rat. The study of parotid glands treated with isoproterenol was originally undertaken in the early 60's as a possible model system for studying the pathobiochemistry of cystic fibrosis (CF), since CF was classified earlier as a "generalized exocrinopathy" and often involved enlargement of the salivary glands (Mehanso, 1987). Repeated administration of isoproterenol to rats causes marked and rapid hyperplasia and hypertrophy of both the parotid and submandibular glands. It has also been shown to increase the rate of DNA, RNA, and protein synthesis. Morphological observations have also shown changes in the contents of acinar secretory granules, including a band of protein on electrophoresis not seen in untreated saliva (Robinovitch, 1977). This band represents a series of unusual proteins containing 25-45% proline, called the proline-rich proteins (PRPs) which appear to be induced in response to chronic isoproterenol stimulation and have been isolated from both human and parotid parotid glands and saliva. The changes resulting from chronic treatment with isoproterenol, an agonist which is non-specific for ß-receptor subtype, have been shown to be mediated through $ß_1$ receptors.

While a regiment of very high doses of the $ß_2$ agonist terbutaline were able to produce the characteristic changes, only the use of $ß_1$ antagonist atenolol could prevent these changes, while the $ß_2$ antagonist butoxamine could not (Schneyer, 1985).

1.4 Acidic Epididymal Glycoprotein (AEG)

1.4.1 History and Characterization

Acidic Epididymal Glycoprotein (AEG) is a 31,700 molecular weight protein that was originally purified from rats in 1978 (Lea, 1978). It is the first epididymal glycoprotein to be isolated and characterized. It is a major secretory protein of the epididymis which accounts for 2-3% of the total soluble protein. AEG qualifies as a glycoprotein because it binds specifically to Concanavalin-A, a conclusive test for carbohydrate moieties (Lea, 1978). The carbohydrate content of AEG is 7.5%, mainly hexoses (73%). It contains a high content of acidic amino acids, aspartic and glutamic acid, (27.5%), with a low isoelectric point, indicating that it is an acidic protein (Lea, 1981).

AEG is also known by several other names in the literature. There is satisfactory evidence to indicate that these are, indeed, the same protein. A series of epididymal proteins B,C, D, and E, named based on their migration position ahead of albumin were among the earliest proteins described in the epididymis. D and E appear to be identical to AEG (Brooks, 1980). Other names for this glycoprotein which appear in the literature include 32 K protein (Wong, 1982), protein IV (Jones, 1980), and sialoprotein (Faye, 1980).

1.4.2 Epididymal Localization

Precise localization of the protein within the epididymis has been determined by several different methods. In one study, the epididymis was divided into five segments from caput to cauda, homogenized, and quantified for AEG using immunoelectrophoresis (Lea, 1978). This showed that AEG increased in concentration from caput to cauda, the direction of sperm flow. Immunoperoxidase localization, performed on a single longitudinal section cut through the entire epididymis, allowed for more precise localization of AEG. Between the initial segment and the main portion of the caput, there is a sudden increase in the number of positive staining epithelial cells as well as a sudden appearance of AEG in the lumen. Higher magnification of the caput indicates that AEG appears in the supranuclear region of principal cells and is found coating the microvilli as well as sperm in the lumen. Nearing the end of the cauda however, the principal cells are free of AEG-staining except for the microvilli, while the clear cells are stained heavily (Lea, 1978). Using *in situ* hybridization of epididymal sections to a cRNA probe, weakly positive cells were observed in the proximal caput, then became strongly positive throughout the rest of the caput and through the cauda. Every principal cell appears to express AEG/DE transcript in the strongly positive regions (Douglass, 1991). Using a cDNA radiolabeled probe, Charest et. al. demonstrated similar levels within the caput, corda, and cauda epididymis by northern blot analysis (Charest, 1988). Recently our laboratory, in collaboration with Dr. Arthur Hand of the University of Connecticut, has localized AEG in the epididymis by electronmicroscopy. (See Figure 5.) AEG is visualized in the secretory vesicles of the principal cells of the epididymis, but not in the other epithelial cells, connective tissue cells, or smooth muscle cells.

An Electronmicrograph of AEG in the Epididymis

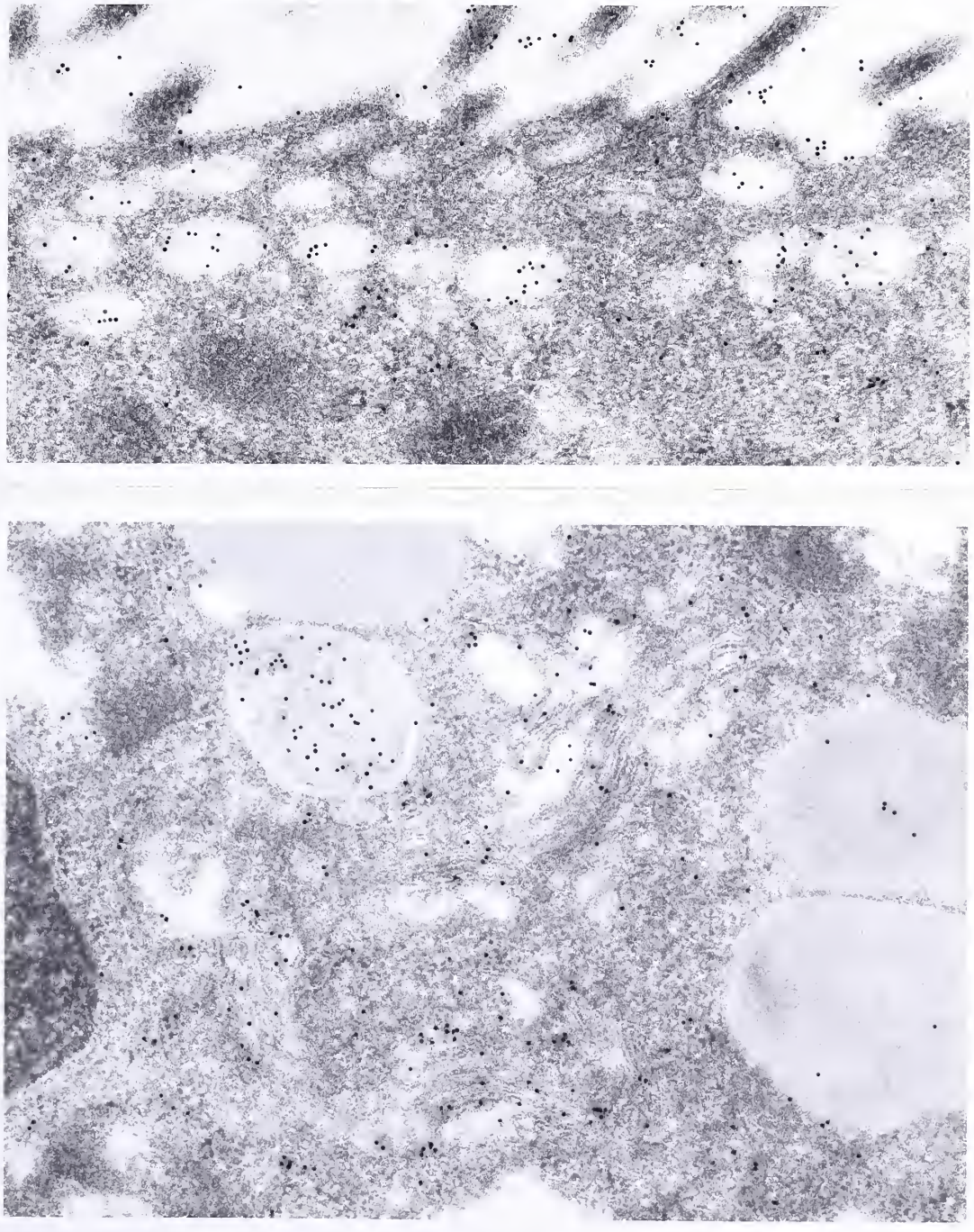


Figure 5: Immunoglobulin-labeled gold particles demonstrate AEG staining in the Golgi body and secretory vessels of principal cells of the epithelium of the epididymis and associated with the microvilli in the lumen. Magnification x56,000.

1.4.3 Sequence and sequence homology

Two different mRNA species of 1100 bases (b) and 1600 b were detected in the epididymis by northern blot hybridization. The distribution of these mRNA species among animals suggests that these represent two alleles for AEG (Charest, 1988). Two full length AEG cDNA clones have been isolated from a rat epididymal cDNA library , sequenced, and characterized. (See Appendix 1) The cDNA's correspond in size to the two RNA species seen by Northern blot hybridization. These clones have identical 5' untranslated and coding regions, but differ in their 3'-untranslated region (Charest, 1988). The cDNA sequence of the epididymal protein D/ E has an identical sequence to this smaller clone (Brooks, 1986).

The predicted amino acid sequence of 246 amino acids matches that of the known AEG sequence. The first nineteen amino acids of the N-terminal region are hydrophobic, which suggests the presence of a signal peptide. The carboxyterminal region is cysteine-rich (Charest, 1988). A computer search of protein libraries has revealed homology of the carboxyterminal region of AEG with ferredoxin, a metal binding protein (Charest, 1988). Several investigators have noted homology between the sequence of AEG and that of certain proteins of known function. Using protein sequence data bases, Brooks observed homology between a region of AEG's amino acid composition and the protein carboxypeptidase Y (Brooks, 1986). AEG also shares sequence homology of the carboxyterminus with the metalloproteins rubredoxin and aspartate transcarbamoylase. It is possible that the AEG might function in sperm maturation by its ability to bind metal (Charest, 1988). A testis-specific gene, Tpx, which codes for a protein of unknown function and is expressed by both mice and humans, shares 64.2% nucleotide and 55.1% amino acid sequence homology with AEG (Kasahara, 1989).

1.4.4 Function

Only a few investigations have focused on possible functions of AEG. Immunoperoxidase staining has shown that AEG binds to sperm in the caput and remains bound to sperm throughout the epididymis (Lea, 1978). Immunofluoresence microscopy using a double-antibody procedure was used to localize AEG on the sperm surface. A restricted pattern of strong fluoresence was seen on the post-acrosomal sperm head (Brooks, 1983). Another study looked at a similar mouse epididymal protein; a 29kD protein which has 69% homology with AEG and to which antibodies cross react with AEG in epididymal fluid. A technique of *in vivo* injection of ^{35}S-methionine was used to study the interaction of epididymal secretory proteins with spermatozoa. The 29kD protein was found bound to the sperm membrane both immediately and after seven days (Vreeburg, 1991). Its presence attached to the sperm membrane throughout much of its transit through the epididymis has led several investigators to suggest that AEG may be involved in the maturation of spermatozoa.

Only one study examined the possible role AEG has on fertility of sperm from the cauda epididymis. The presence of anti-AEG serum, but not normal rabbit serum, resulted in an 85% loss of the fertilizing ability of sperm by *in vivo* insemination. However, this approach could not rule out the possibility of steric hindrance to gamete recognition caused by the presence of immunoglobulins on the cell surface (Cuasnicu, 1984).

1.4.5 Regulation

The role of androgens in the regulation of AEG has been examined by monitoring AEG levels in the epididymis of castrated rats. Several studies have demonstrated that AEG mRNA levels declined steadily after castration over one week. Testosterone replacement returns AEG mRNA levels to baseline with 72 hours after injection (Charest, 1988) ; (Brooks, 1987). Another study used an agent which causes transient depletion of sperm to test the hypothesis that spermatozoa or a sperm-associated testicular factor may be involved in the regulation of AEG gene expression. Busulfan is an alkylating agent which causes the depletion of sperm from the epididymis approximately 7 to 12 weeks following administration. While another protein in this study, proenkephalin, showed a dramatic decrease in mRNA levels, AEG mRNA levels were unaffected by the loss of spermatozoa (Douglass, 1987).

1.4.6 Development

The question of regulation of gene expression of AEG has also been approached by examining the developmental time course and comparing it to the presence of epididymal androgens and androgen receptors. In a study by Charest et al., AEG mRNA was detectable at 1 day of life, but significant concentrations were not seen until day 20. Concentrations increased steadily, with the most rapid rise from day 20 to 35; adult levels were reached at day 45. High levels of androgens within the epididymis were detected as early as day 10. Androgen receptor mRNA is present at all ages. Since AEG does not reach significant levels until day 20, despite the presence of androgens and AR before this age, androgens appears not to be sufficient to activate AEG transcription (Charest, 1989). This developmental pattern has

also been seen by other investigators working with AEG; although they were unable to detect mRNA's before day 20, most likely secondary to technique differences (Brooks, 1987); (Douglass, 1991).

1.4.7 Tissue Distribution

One study examined various rat tissues by Northern blot analysis with AEG cDNA. Liver, spleen, testis, seminal vesicle, dorsal prostate, epididymis, and ductus deferens were analyzed, but only the latter two were positive. However, the levels of AEG in the ductus deferens were one tenth the level in the epididymis (Charest, 1988). Another study, done by Western blot, was unable to detect a signal from skin, brain, liver, heart, skeletal muscle and testis. A cross-species study of the epididymis found smaller but cross-reactive proteins in mouse and guinea pig but not from rabbit or bull (Brooks, 1986). A year later, the same investigators used slot-blot cDNA hybridization to examine the above tissues plus uterus, mammary gland, and salivary gland in the rat. A weak hybridization signal was detected from the salivary gland preparation (Brooks, 1987).

The discovery of a weak signal from mRNA from a mixed salivary gland preparation led to a more specific study of the individual salivary glands in our laboratory. Northern blot hybridization of AEG mRNA in the salivary glands revealed that AEG was present in the parotid but not the submandibular or sublingual glands (Charest, 1991). When a polyclonal antiserum to epididymal AEG was incubated with a Western blot of saliva derived from the parotid gland of rats, a major band of 31,000 Mw was revealed. Electron microscope immunogold labeling of the parotid gland with AEG antiserum demonstrated strong reactivity with acinar secretory granules, but no labeling of duct cells or any other cell type. (See Figure 6.)

An Electronmicrograph of AEG in the Parotid Gland

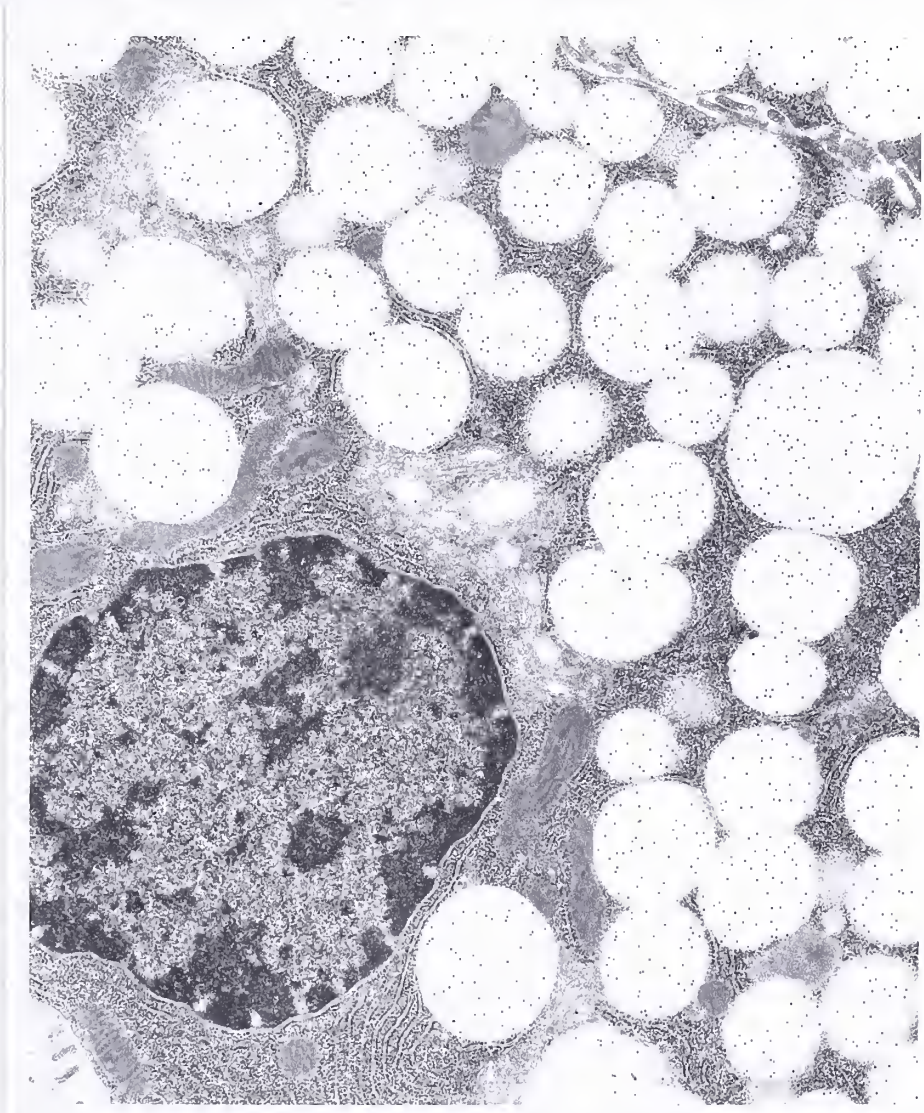


Figure 6: Immunogold labeling of polyclonal antibody to AEG in the acinar cells of the rat parotid gland showing extensive secretory gramule labeling. Magnification x18,000.

Northern blot hybridization with the epididymal AEG cDNA probe have revealed two parotid transcripts of 1.4 and 0.9kb, compared to epididymal transcripts of 1.6 and 1.1kb (Charest, 1991). Sequence of the parotid AEG cDNA has revealed that the coding region is 100% homologous to the coding region of epididymal AEG cDNA. Preliminary experiments suggest that differences in the 5' untranslated region accounts for the differences in length. (Charest, Beck, Naiman, and Hand; manuscript in preparation) In addition, we have shown that both male and female rats express AEG in the parotid gland. (See Figure 7) The findings of equivalent levels in male and female animals led us to speculate whether the regulation of AEG mRNA in each tissue is different.

1.5 Statement of purpose

The expression of AEG in the epididymis and the parotid gland, which differ in embryological origin (mesodermal vs. ectodermal) and function creates a unique situation in which to study tissue-specific gene expression. The purpose of this study is to test the hypothesis that the hormonal control of AEG gene expression in the epididymis differs from that in the parotid gland.

Comparison of AEG mRNA Levels in the Parotid Gland and the Epdidymis

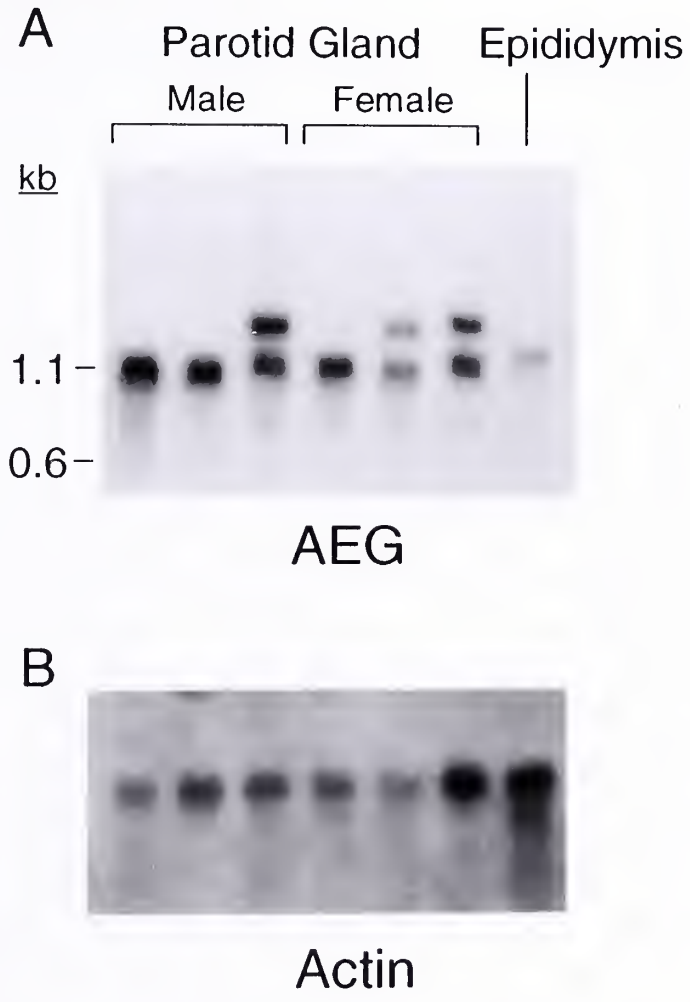


Figure 7: Parotid gland AEG mRNA levels from male and female rats are compared to the levels in the epididymis. The rats shown are either heterozygous or homozygous for the smaller message. (A) AEG mRNA (B) Actin mRNA (shown as control for amount of RNA present in each lane) Note that the amount of RNA from the epididymis is greater than from the parotid gland, yet the signal is much less intense. This suggests that AEG mRNA is a greater portion of the total RNA pool in the parotid gland than in the epididymis.

2.0 Materials and Methods

2.1 Tissue Isolation

Sprague-Dawley rats (Simonsen Laboratories, Gilroy, California) were used in all experiments (except for studies requiring TFM rats) as they have been shown in previous studies to be homozygous for the smaller mRNA species of both the epididymis and parotid species (Charest, personal communication). King Holtzman TFM rats and their normal male siblings were kindly provided by Dr. Kathie Olsen (The National Science Foundation). Rats were kept under routine conditions of temperature and light with ad lib access to pelleted food and water. For certain experiments (detailed in Results, Section 3.0), rats received injections of hormones prior to collection of tissues. Animals were anesthetized with ether, and killed by cardiac puncture. The parotid gland and epididymis were dissected from surrounding tissue, rapidly frozen in liquid nitrogen, and stored at -70°C. All protocols involving animals were performed within the guidelines set by the Yale Animal Care and Use Committee.

2.2 Castration

For experiments where castration was required, animals were anesthetised prior to surgery by intramuscular Ketamine, injected at a dose of 1ml/kg body weight. A midline abdominal incision was made and the contents of the scrotal sac were elevated into the abdomen. The testicular artery was tied shut with a suture before removal of the testes. The epididymides were returned to the scrotal cavity and the abdominal incision closed with staples.

2.3 Isolation of Total RNA

Frozen epididymis or parotid gland was pulverized under liquid nitrogen and homogenized in 4M guanidine thiocyanate using a Brinkman polytron probe sonicator. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was layered over a cesium chloride gradient in a Ti 50 Rotor in a Beckman Ultracentrifuge for 16 hrs at 35,000 rpm. The pellet was resuspended in H_2O and Na acetate and ethanol precipitated at -20°C. (Chirgwin, 1979). This precipitate was redissolved in water and purity and concentration were measured by UV absorbance at 260λ and 280λ. 40 µg /µl RNA is equal to 1 unit A_{260}. The quality of the RNA was checked for accuracy by loading a 2 µl aliquot onto a 1% agarose gel with ethidium bromide and running with 0.5 x TBE; the gel was examined under UV light to look for degredation.

2.4 Use of cDNA Probes

The 5' EcoR1 fragment of the 1500 base pair cDNA for AEG, which recognizes both the 1100 and 1600 base mRNA species of the epididymis and the 1400 and 900 base mRNA species of the parotid, were used for the Northern blot analysis (Charest, 1991). The AEG cDNA was labelled by random priming (Feinberg, 1983) with ^{32}P dCTP to a specific activity of 10^8-10^9 cpm/µg. Incorporated ^{32}P dCTP was separated from free by running the reaction mixture through a 5ml column of Sephadex beads and removing aliquots. The radioactivity of the aliquots was assessed in Ecoscint scintillation solution (National Diagnostics) in a Packard Tri-Carb 1600CA Liquid Scinitillation Analyzer. Chicken actin cDNA was also prepared in this same manner.

2.5 Northern Blot Analysis

Northern blot was performed with modifications from Sambrook's Laboratory Manual (Sambrook, 1989). An aliquot of total RNA (10μg) was denatured with glyoxal and dimethylsulfoxide for 1 hr at 50°C. RNA samples were loaded onto a 1.2% agarose gel and electrophoresed in circulating 10mM Na Phosphate (pH 6.8) buffer. Denatured ^{32}P-labeled DNA markers, ϕX174 Hae III and λ Hind III (New England Biolabs, Beverly, Massachussets) were loaded onto each gel for later estimation of RNA size. The RNA was then transferred overnight onto Biotrans membrane (ICN, Irvine California) and subsequently fixed by baking at 80°C for 1 hour and exposure to UV light. The membrane was prehybridized in 50% formamide, 50mM $NaPo_4$, pH 6.5, 5xSSC (SSC = 0.15M NaCl, 0.015 M Na citrate), 5x Denhart's solution, 250 μg/ml boiled sonicated salmon sperm DNA (BSSS), 0.5% sodium dodecyl sulfate (SDS) and 1% glycine at 42°C for 2hrs. The prehybridization solution was removed and the membrane was hybridized overnight in 50% formamide, 20mM $NaPo_4$, pH 6.5, 5xSSC, 1x Denhart's solution, 100μg/ml BSSS DNA, 0.5% SDS, and 10% dextran sulfate with the labeled AEG cDNA probe (1×10^5 cpm/ml hybridization fluid) at 42°C. The membrane was subsequently washed three times in 2xSSC , 0.1% SDS at room temperature and twice in .1x SSC, 0.1% SDS at 50°C and exposed to x-ray film at -70°C. To check for variation due to RNA loading and/or transferring differences, the radioactive probe was later removed from the membrane by incubating at 65°C for 1 hr in Stripping Buffer (50% formamide, 10mM Na phosphate, pH 6.8). The filter was then prehybridized and rehybridized as described above with radiolabeled actin cDNA. In some cases, band intensity on autoradiographs was quantified by densitometry using a LKB Ultroscan XL.

2.6 DNA isolation

Frozen tissue was pulverized under liquid nitrogen and mixed with DNA lysis buffer (50mM Tris, 10mM EDTA, 100mM NaCl, 0.5% SDS, 100µg/µl Proteinase K) for 3 hrs at 50°C. The DNA was extracted with phenol/chloroform and precipitated in ETOH. The DNA strands were dissolved in TE (10mM Tris, pH 8, 1mM EDTA) and placed in dialysis tubing. They were then dialysed against TE to a 10^{-5} dilution. The dialysate was collected and the DNA concentration determined by UV spectrometry at 260λ; 50 µg/µl DNA is equivalent to 1 A_{260} unit (Sambrook, 1989).

2.7 Acknowledgement of Participation of Others

In keeping with the new Yale University School of Medicine policy, this section within the method portion of this thesis briefly outlines the extent of my direct involvement and that of others in the procedures, methods, experiments, and generation of data. In close collaboration with Dr. Charest, I arrived at the hypothesis to be examined and the experimental protocols to be implemented. For the castration experiments, I closely observed Dr. Charest and Mr. Ralph Garcia (research assistant in the pediatric respiratory division) as they performed the first two surgeries and then I castrated all the remaining animals under Mr. Garcia's immediate supervision. Dr. Arthur Hand (University of Connecticut Health Center Dept. of Orthodontics and Pediatric Dentistry) assisted with the parotid gland dissections. He dissected all of parotid glands of the immature animals and during the isoproterenol study, and taught me the techniques so that I was able to dissect the parotid glands from the other adult rats. I performed all of the epididymal dissections.

I prepared all of my own reagents used in these experiments. I performed on my own all RNA and DNA isolations, cDNA probe labeling, Northern blot hybridizations, film developing, and densitometry as outlined above.

3.0 Results

3.1 Developmental Profile of AEG in Parotid and Epididymis

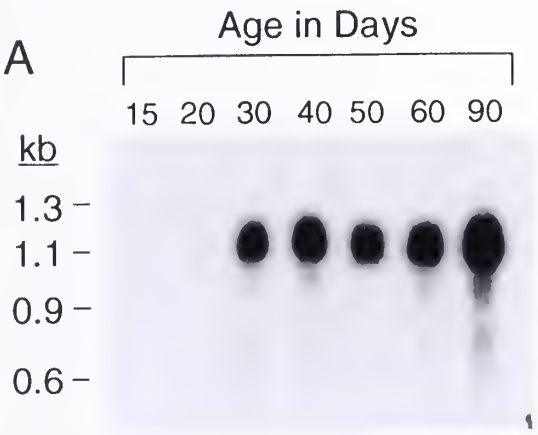
During the course of development, tissue-specific proteins progressively change in amount and number. Eventually, the pattern of protein synthesized by a developing tissue comes to resemble that of adult tissue. The mechanisms underlying the differentiation of tissues must produce changing patterns of individual gene activation and deactivation (Darnell, 1986). Thus the developmental profile of a tissue-specific protein is important in understanding its regulation. The developmental profile of AEG mRNA in the epididymis has already been well characterized. (see section 1.4.6) To examine the developmental profile of AEG in the parotid, normal male rats were sacrificed at 15, 20, 30, 40, 50, 60, and 95 days of age. Because of the small sizes of the glands at the earlier ages, multiple glands were pooled to reach the target starting frozen weight of 0.5g. Northern blot analysis is shown in Figure 8 A and B. With prolonged exposure, a faint signal is seen at day 20, which is evident in figure 8 C. The greatest increase is shown between day 20 and day 30. Between days 30 - 60, no change is seen in AEGmRNA levels. The Actin cDNA hybridization demonstrates that the 15 day age lane has twice the mRNA of the other lanes yet still does not show any hybridization to AEG cDNA.

To more closely compare the regulation of AEG in both tissues, the parotid gland and epididymal AEG mRNA levels were compared from the same animals at ages 15, 20, 25, and 30. Figure 8 C shows that the initiation of expression occurs within a few days developmentally in the two tissues. In general, the developmental pattern of expression of AEG in these two tissues is similar.

Developmental Profile of AEG in the Parotid Gland and in Comparison to the Epididymis

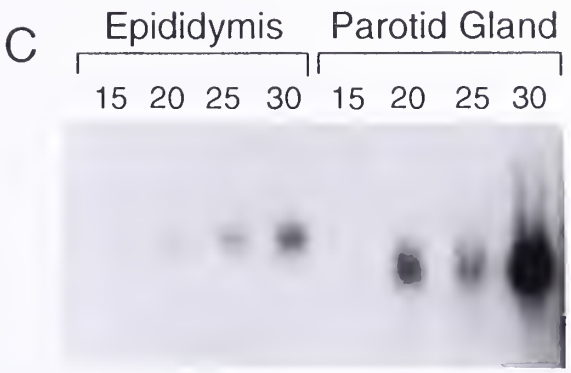
Figure 8: Autoradiograph of Northern blots from Developmental Studies. (A) Hybridization of AEG ^{32}P-cDNA probe shows Parotid AEG mRNA levels at 15, 20, 30, 40, 50, 60, and 90 days of age. (B)Hybridization of Actin ^{32}P-cDNA probe shows Actin mRNA levels The first lane (age 15) is loaded with 20 μg of RNA instead of 10μg. (C) Comparison of early developmental profile of AEG in the parotid gland and epididymis at 15, 20, 25, and 30 days of age. A signal is detectable in the parotid at 20 days of age. However, the 30 day lane is overexposed (compare the signal intensity to the equivalent lane in 8 A) in order to visualize the signal from the younger animals.

Parotid Gland



AEG

Actin



AEG

3.2 Effects of Androgens on AEG in the Parotid Gland

As the hormonal regulation of epididymal AEG by androgens has been well established, (see section 1.4.5) the initial phase of investigation was to determine whether there was a similar control of gene expression of parotid AEG mRNA expression. To examine this possibility, adult female rats were treated with daily injections of testosterone propionate for 24 or 48 hrs and compared with untreated controls. Figure 9 shows the results of this experiment, analyzed by Northern blot technique. No difference was seen in AEG mRNA levels of the testosterone-treated females compared to the untreated female controls.

Another approach to the question of androgen regulation of AEG in the parotid gland is presented in Figure 10, where the levels of parotid AEG mRNA in androgen resistant (Tfm) rats are compared to that of their normal male siblings. Once again, no difference in parotid AEG levels is seen, suggesting that androgen receptors are not required for AEG gene expresion in the parotid gland.

The results of the castration experiment are presented in Figure 11. Northern blot analysis of RNA isolated from the parotid glands and epididymis of rats two weeks after castration and intact controls show no change in parotid AEG mRNA levels while an approximate 50% decrease was seen in the epididymis. Administration of 2mg IM testosterone proprionate to the two remaining castrated rats restored AEG mRNA epididymal levels to control levels but again had no effect on AEG mRNA in the parotid gland. Taken together, these three experiments strongly indicate that androgen is not required for AEG expression in the parotid gland.

Parotid AEG mRNA Levels in Testosterone-Treated Females

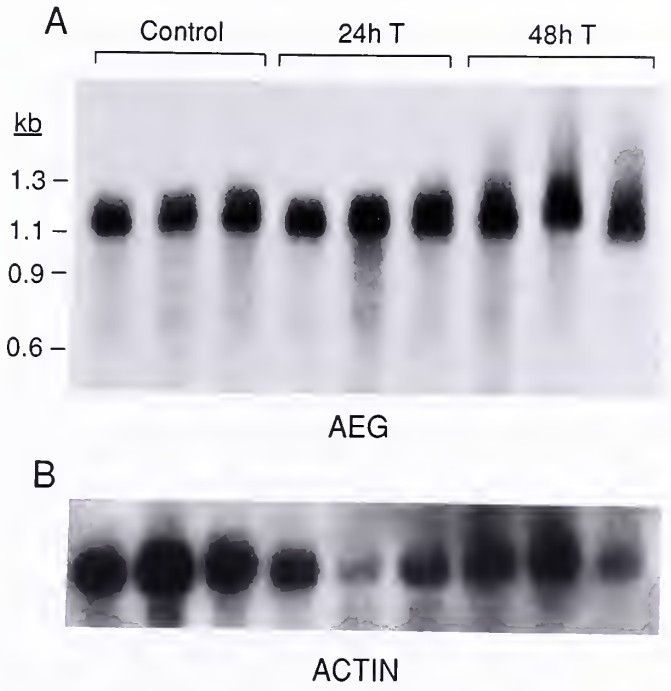


Figure 9: Autoradiograph of the Northern blot for testosterone-treated females. Six adult female rats were injected intramuscularly with 2mg testosterone propionate. Animals were killed 24 or 48 hr after hormone treatment. Three control animals received no treatment. (A) Hybridization of AEG ^{32}P-cDNA probe showing AEG mRNA levels (B) Hybridization of Actin ^{32}P-cDNA probe showing Actin mRNA levels

Parotid AEG mRNA Levels in Tfm Rats

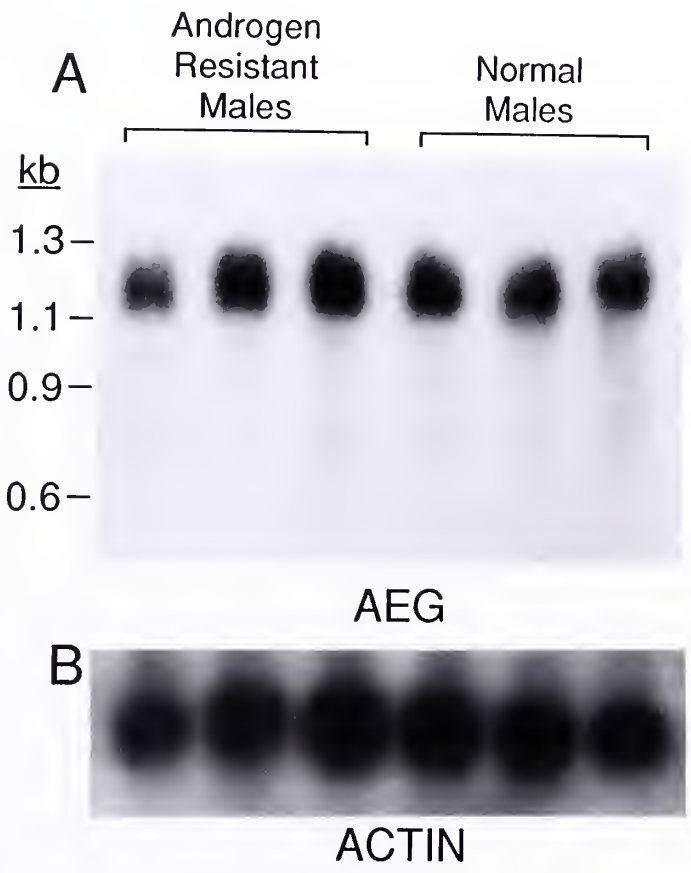


Figure 10: Autoradiograph of Northern blot showing a comparison of AEG mRNA levels in the parotid glands between Tfm rats and their non-affected brothers. (A) Hybridization of AEG ^{32}P-cDNA probe showing AEG mRNA levels (B)Hybridization of ^{32}P-cDNA probe showing Actin mRNA levels

Effects of Castration and Testosterone Replacement on AEG mRNA levels in the Parotid Gland and Epididymis

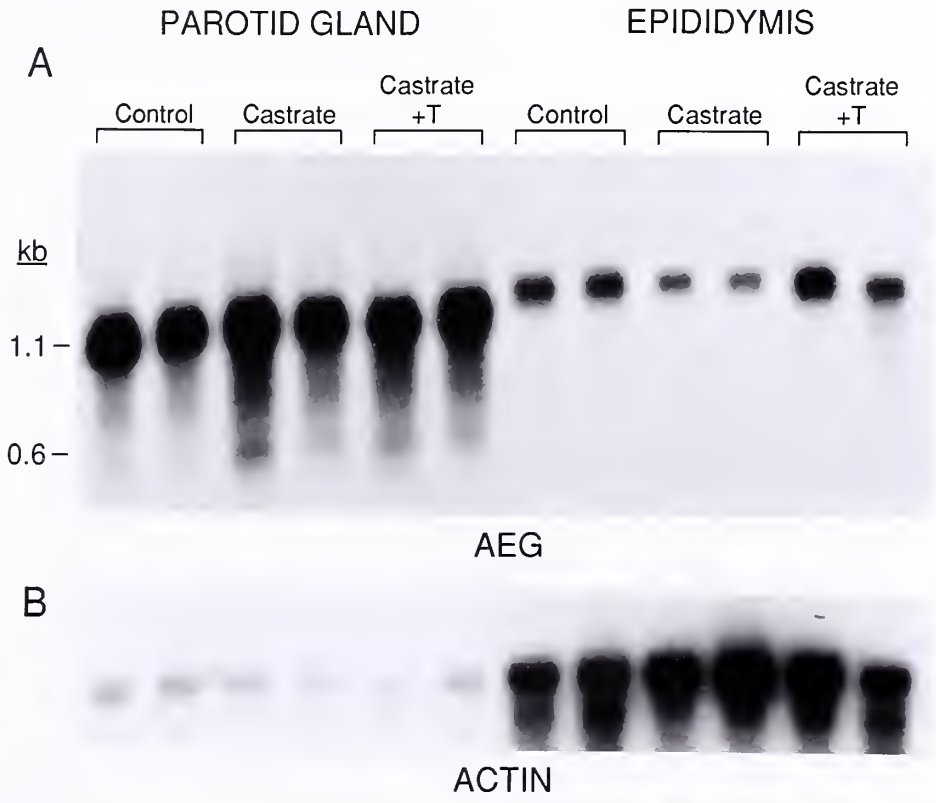


Figure 11: Autoradiograph of Northern blot from the castration study. Four adult male rats were castrated by an abdominal approach. Two animals were killed 7 days after castration. The other 2 animals received a single injection of 2mg testosterone propionate 7 days post-castration and were killed 72 hours later. Two control animals received no treatment. Each lane contains RNA isolated from two animals. (A) Hybridization of AEG ^{32}P-cDNA probe showing AEG mRNA levels in parotid and epididymis (B) Hybridization of Actin ^{32}P-cDNA probe showing Actin mRNA levels in parotid and epididymis. Actin is known to be a more abundant mRNA species in epididymal RNA pools compared to parotid.

3.3 Effects of Isoproterenol on AEG in the Parotid Gland and Epididymis

The effects of chronic isoproterenol administration on AEG in both tissues was investigated because ß-adrenergic agonists are known to be major regulators of parotid gland gene expression. Six adult male rats were given daily intraperitoneal injections of 5mg of isoproterenol for 7 days. Tissue for analysis was either taken immediately, or after a recovery period of five days during which time the animals received no treatment. Figure 12 shows the Northern blot analysis of this experiment. Parotid AEG mRNA levels were reduced to approximately 10% of untreated control animals while no change was observed on epididymal AEG levels. No significant change was noted in AEG mRNA levels following a five day recovery period.

3.4 Biochemical effects of Isoproterenol in the parotid gland and the effects on measured levels of AEG mRNA

Because chronic isoproterenol treatment is known to effect many biochemical parameters within the parotid gland, including increasing RNA and DNA synthesis (see section 1.3.6) , the interpretation of Northern blots from rats given IPR treatment is not straightforward. Since Northern blot analysis uses a fixed amount of RNA regardless of the total volume of the RNA preparation, it is a relative, not an absolute, technique. To address the possible factors influencing the Northern blot results, changes in parotid gland weight, DNA, and RNA, were examined in control and IPR treated rats. These results are presented in Table 1. Of note, both gland weight and total DNA increase 3 fold with IPR-treatment. There was a more pronounced effect on total RNA levels, with treated animals having a 7.4 fold increase

Effects of Isoproterenol on AEG mRNA Levels in the Parotid Gland and Epididymis

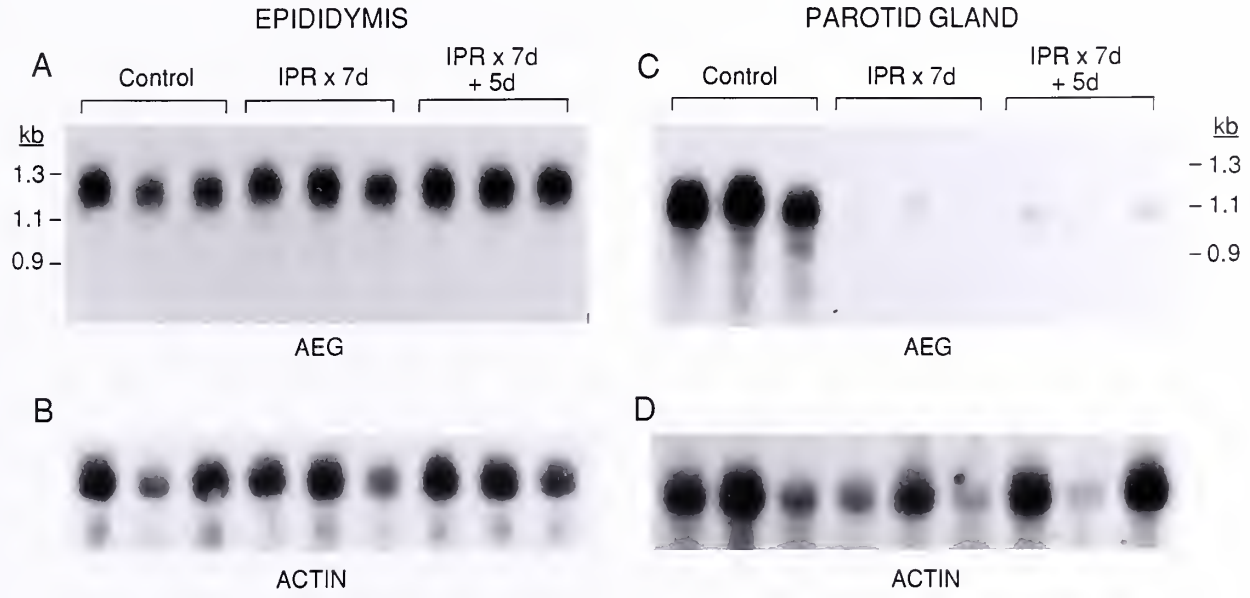


Figure 12: Autoradiograph of Northern blot from the Isoproterenol (IPR) study. Three adult male rats were given daily intraperitoneal injections of 5mg of isoproterenol in 1 ml physiologic saline for 7 days. Another group of 3 animals received 7 days of treatment and then were allowed to recover for 5 days. A control group of three animals received no treatment. (A) Hybridization of AEG ^{32}P-cDNA probe shows AEG mRNA levels in parotid (B) Hybridization of AEG ^{32}P-cDNA showing AEG mRNA levels in epididymis (C) Hybridization of Actin ^{32}P-cDNA shows Actin mRNA levels in the parotid (D)Hybridization of Actin ^{32}P-cDNA shows Actin mRNA levels in the epididymis

Table 1

Biochemical Assays on Parotid Glands of Control and Isoproterenol-treated Rats[1]

	Control	IPR-treated	P	Fraction
Gland Weight (mg)	342 ± 23	1073 ± 71	<.001	3.1
Total DNA (mg/gland)	0.54 ± 0.1	1.73 ± 0.6	<.05	3.2
Total RNA (mg/gland)	1.94 ± 0.3	14.29 ± 1.06	<.001	7.4
RNA:DNA Ratio	3.6	8.3		2.3

[1] N = 3 rats in each group, except for gland weight were N = 9

[2] Fraction that IPR represents of the control. Value < 1.0 represents a decrease from the control levels.

Table 2

AEG mRNA levels by Densitometry[1] in Parotid Glands of Control and Isoproterenol-treated Rats

	Animal	Control	Animal	IPR	Fraction
AEG mRNA /	1	4.979[2]	4	0.749	
10µg RNA	2	8.253	5	0.606	
	3	6.313	6	1.034	
Average[3]		6.52 ± 1.7		0.79 ± 0.22	.12
Total AEG[4]		1263.9		1137.0	.90
TotalAEG/mg DNA		2340.6		657.2	.28

[1] AEG mRNA levels measured by densitometry area are in absorbance units (AU) x mm

[2] Each value reported is the average of measurements made from duplicate loading of samples on the gel.

[3] $P < 0.01$

[4] Total AEG mRNA levels determined by mRNA in AU x mm/10µg RNA x Total mRNA (as determined in Table 1 for control and IPR-treated parotid glands)

over controls. Table 1 also shows that there is a 2.3 fold increase in the total RNAper mg DNA in the IPR-treated group. The three-fold increase in both gland weight and DNA suggests that DNA is a reasonably good estimate of cell number. Results were found to be statistically significant using the Student's T-test (Freedman, 1980).

Because there is a 2.3 fold increase in RNA with respect to DNA, with IPR-treatment, the decrease in AEG mRNA levels do not necessarily reflect a decrease in transcription rate. From the total RNA isolated from the control and IPR-treated animals, another Northern blot analysis was performed for the purpose of quantifying the AEG mRNA levels by densitometry. (See figure 13) Table 2 shows the results of the AEG mRNA levels as determined by densitometry. AEG mRNA levels were reduced to 12% of controls. Total AEG mRNA levels are decreased in the treatment group in spite of the seven-fold increase in total RNA. When total AEG mRNA levels are taken in terms of cell number, as estimated by changes in total amount of DNA, IPR-treated AEG mRNA/cell is reduced to 28% of control per cell, an estimated 3.5 fold decrease. If the reduction was due soley to the dilution of AEG mRNA in a larger pool of total RNA, the expected reduction would be 43%. The difference between the expected and observed values strongly suggest that AEG transcription is directly inhibited by IPR.

Effects of Isoproterenol on AEG and Actin mRNA Levels in the Parotid Gland

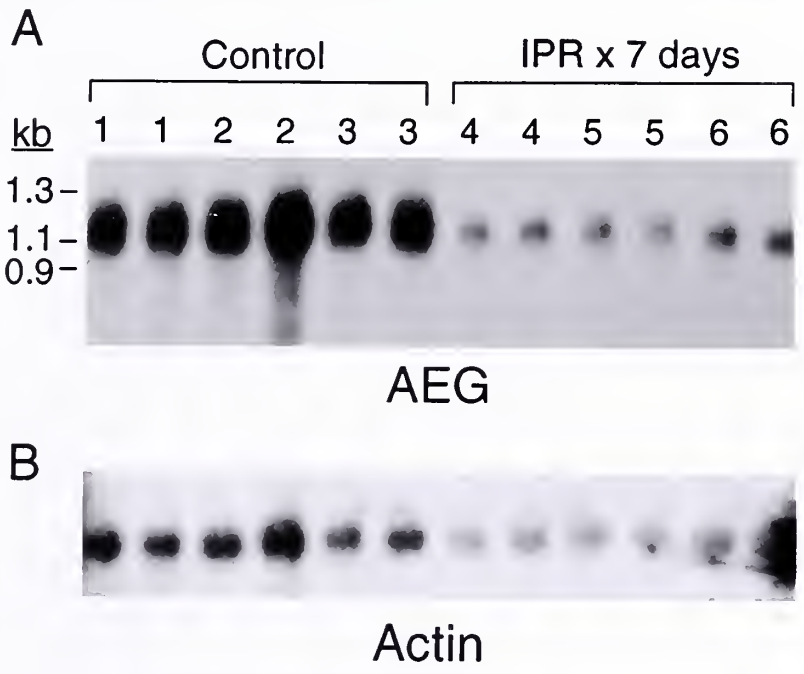


Figure 13: Autoradiograph of Northern blot from the repeat Isoproterenol study used to quantify changes by densitometry. Three adult male rats were given daily intraperitoneal injections of 5mg of isoproterenol in 1 ml physiologic saline for 7 days. A control group of three animals received no treatment. Duplicates were run of each10µg mRNA sample on the gel. (A) Hybridization of AEG ^{32}P-cDNA probe for shows AEG mRNA levels B) Hybridization of Actin ^{32}P-cDNA shows Actin mRNA levels. Densitometry results are presented in Table 2.

4.0 Discussion

Several conclusions can be drawn from these experimental results. First, the developmental profile of AEG in the parotid gland and the epididymis is similar; the initiation of significant expression of AEG in the two tissues is occurs around the same time, suggesting that a common circulating factor may be involved. Secondly, while AEG mRNA has been previously known to be regulated by androgens, which was again demonstrated during the castration study (see figure 11), androgens have no effect on the regulation of AEG in the parotid gland. Finally, isoproterenol decreases the expression of AEG in the parotid gland but not in the epididymis, which is a true effect, not a dilution of AEG mRNA in a more abundant pool of mRNA's.

4.1 Developmental Expression

Both parotid and epididymal AEG show the greatest increase in overall AEG mRNA transcription between day 20 and 30, although the pattern is not identical. Developmental expression of AEG mRNA expression in the mouse submandibular gland also shows the sharpest increase between day 25 and 30 (Mizuki, 1992). This suggests that there may be a circulating factor which becomes available around day 20, which can account for the developmental increase in transcription. In the parotid gland, tissue-specific changes in protein and glycoprotein synthesis occur shortly after birth and again at the time of weaning. Incorporation of [^{3}H]thymidine, a measure of DNA synthesis, reaches its highest after birth in the parotid gland and then declines. However, incorporation of [^{14}C] leucine, a measure of protein synthesis, increases dramatically between days 21 and 28. The α-amylase first reaches significant levels during this developmental period and a new protein band of 25,000 molecular weight is first detected (Humphreys-Baher, 1982). It is

possible that the increase in AEG mRNA represents an overall tissue-wide increase in transcriptional activity in the parotid gland which is occurring at the developmentally significant time of weaning. In the epididymis, this is a similarly developmentally significant time period with the formation of the blood-testis barrier and the appearance of sperm and sperm-associated fluid in the lumen of the epididymis. (See section 1.2.3) AEG has a similar timetable for expression to that of other androgen-dependent epididymal proteins, but the precise mechanism is still unknown. It is presently not known how the induction of androgen-dependent genes is controlled during development. Expression of these genes usually occurs before puberty but long after the establishment of significant circulating levels of androgens. Possibly a tissue specific factor, perhaps produced by the fully differentiated epididymis, or a testicular factor, is necessary for the initiation of high levels of AEG.

4.2 Androgen is not involved in the regulation of parotid AEG mRNA

Several experiments (shown in Figures 9-11) addressed the question of the role androgens have in the regulation of AEG in the parotid gland by manipulating circulating androgen levels through different methods. Female rats with normally low levels of circulating androgens were given doses well above their physiological levels. Rats which lack a functional androgen receptor and therefore are resistant to the effects of androgens were compared to males with normal androgen receptors. Male rats were deprived of their major site of synthesis of androgens by castration and had levels restored by replacement doses of testosterone. None of these measures influenced parotid AEG mRNA levels. However, androgen-regulated gene expression does occur outside of the male reproductive tract. For instance, the transcription of the major urinary proteins of the mouse in the liver, and ornithine decarboxylase

and alcohol dehydrogenase in the kidney are known to be androgen regulated (Berger, 1989). But even more interesting is the existence of an acidic epididymal glycoprotein molecule in the epididymis and salivary gland of the mouse which appears to be androgen-regulated in both tissues.

4.2.1 Expression of AEG in the Salivary Gland in the Mouse is Androgen Regulated

Mouse epididymal AEG has been isolated, found to have amino acid sequence 68% homologous to rat AEG, is associated with sperm in transit, and has the same intraepididymal distribution pattern (Rankin, 1992). Mizuki et al. isolated AEG cDNA clones from both mouse epididymal and submandibular gland cDNA libraries (Mizuki, 1992). A cDNA clone, designated AEG-1, was found in the mouse epididymis, while two clones, AEG-1 (identical to the epididymis) and AEG-2, were found in the submandibular gland. The deduced amino acid sequences of the mouse AEG-1 and AEG-2 were 70% and 62% identical to the rat AEG sequence, respectively. Unlike in the rat (see figure 7), male mice had hybridization signals 5 times stronger than and female mice by Northern blot analysis in the submandibular gland. Administration of testosterone to female mice increase AEG mRNA approximately to male levels by 7 days. Cellular localization by *in situ* hybridization revealed that AEG was expressed in the granular convoluted tubule cells of the submandibular gland, but not the acinar or intercalated duct cells (Mizuki, 1992). Another investigator confirmed the androgen regulation of submandibular AEG expression through Northern blot analysis of castration study of AEG mRNA levels in male mice (Haendler, 1993).

4.2.2 Androgen Receptor Distribution

The question remains as to why androgen regulates AEG in the submandibular gland of the mouse, but has no effect on AEG in the parotid gland in the rat. One answer may lie in the tissue distribution of androgen receptors. As stated in the introduction, one of the most important factors in determining the response of a tissue to a hormone is the presence of a receptor for that hormone in the tissue. All cells are exposed to androgens *in vivo* . But the selective action on target tissues depends on the presence of intracellular receptor proteins which can bind the hormone or its metabolites. In humans, the submandibular and parotid glands of both sexes metabolized androgens. Androgen receptors (AR) have been demonstrated by immunohistochemistry using polyclonal antibodies in the nuclei of human acinar cells of both glands (Laine, 1992). In the rat, immunohistochemical localization of AR has been used to confirm their existence in the epithelial cells of the submandibular gland (Sar, 1990). Audioradiographic localization of AR in rat parotid gland showed a heavy nuclear concentration of radioactivity in most of the serous acinar cells, but not in ductal cells or connective tissue (Stumpf, 1976). In fact, androgen receptors have an almost ubiquitous tissue distribution and are found in select cell types of the prostate, seminal vesicle, epididymis, vagina, uterus, cervix, testis, ovary, kidney, liver, adrenal gland, skeletal and cardiac muscle, pituitary gland, and various regions of the brain in the rat (Takeda, 1989). Androgen receptors are found in the rat parotid gland. An alternate explanation is required to explain the lack of androgen regulation of AEG in the rat parotid gland. One possibility is that androgen receptor complexes plus a tissue-specific factor may be required for activation of AEG transcriptions, and these factors are found in epidiymis and mouse submandibular gland but not in rat parotid gland.

4.3 Isoproterenol Decreases Parotid, but not Epididymal AEG mRNA Levels

The first IPR experiment (shown in figure 12) demonstates a marked decrease in AEG mRNA levels. To address the possibility that this decrease is actually the result of a change in transcription rate, the RNA and DNA levels before and after IPR-treatement were quantified. The biochemical analysis of the change in mRNA levels in isoproterenol-treated parotid glands showed a seven fold increase in total mRNA and a three fold increase in weight and DNA. This agrees favorably with changes in parotid parameters resulting form chronic isoproterenol administration seen by a previous investigator, who found a four fold increase in weight (although this was wet weight, not N_2 frozen weight), a three fold increase in protein and total DNA, and a five fold increase in total RNA (Robinovitch, 1977). However, there is an 8.3-fold drop in the level of AEG mRNA by Northern blot analysis in isoproterenol-treated rats, which is more than could be accounted for by the overall increase in mRNA transcription. The rate of transcription of Actin mRNA in parotid glands stimulated with isoproterenol have been shown to be unchanged (Roberts, 1991). Actin thus serves as a good control for Northern blot analysis, and the slight three-fold decrease seen in the actin hybridization signal in figure 13 probably represents the true order of magnitude for the dilutional effect. Therefore isoproterenol does appear to decrease parotid AEG gene transcription.

4.2.1 Isoproterenol as a Known Inhibitor of Gene Expression

Isoproterenol is a well-known enhancer of expression of several different proteins. (See section 1.3.6) However, few studies have addressed the possibility that ß-adrenergic stimulation can have an inhibitory effect on the expression of genes for secretory proteins of the salivary glands. Several

investigators followed levels of secretory proteins in acinar cell secretory granules of the submandibular gland (SMG) under chronic isoproterenol stimulation conditions by immunogold labeling. Unlike any other protein in this study, the labeling intensities for glutamine/glutamic acid rich proteins (GRP), major secretory proteins of the rat, decreased 74% (relative to controls) after stimulation began (Matsura, 1991). These results have been supported by other investigators who followed GRP mRNA levels in SMG treated with isoproterenol. They found a four and a half fold decrease in mRNA levels of GRP, leading them to conclude that isoproterenol appears to modulate GRP expression by alteration in the steady-state GRP transcript level (Cooper, 1991). In the rat parotid gland, statin gene control was found to be down-regulated by isoproterenol both at the mRNA level through Northern analysis and at the protein level by immunofluorescence microscopy (Ann, 1991).

There is considerably less knowledge about the way in which hormones repress transcription through DNA binding proteins. There is, however, every reason to believe that selective repression is an important mechanism of transcriptional control. There are several proposed mechanisms of repression. In the "prokaryotic competition" model, a repressor protein binds near or at the transcription start site and blocks the interaction of transcription factors, like RNA polymerase, with the promoter. In the "eukaryotic competition" model, transcription is dependent on an upstream promoter, and enhancer and inhibitor proteins compete to bind to overlapping or closely linked sequences in the upstream promoter region. The "quenching " model proposes that the activating (A) and repressing (R) proteins bind to adjacent, non-overlapping DNA sequences and protein-protein interactions between A and R prevents A from contacting the transcription complex. Finally, "direct repression" suggests that the negative control factor directly blocks the activity

of a basal transcription complex (Levine, 1989). These models suggest possible ways isoproterenol could influence AEG expression at the transcriptional level.

Fine tuning of transcription of active genes appears to be achieved in many cases by competition between activators and repressors (Jackson, 1991). Isoproterenol, which leads to cAMP elevations and activation of PKA and stimulation of gene transcription, can also activate a specific repressor protein, phosphorylated by PKA's catalytic subunit and which can also bind to the cyclic AMP response element (CRE). This repressor protein has been well characterized and named cAMP response element modulator (CREM). CREM can form dimers with itself or the enhancer CREB; both dimers will bind to the identical CRE sequence, which is part of the upstream promoter, but they are incapable of activating transcription (the "eukaryotic competition" model). The formation of certain dimer compounds provides a means to inhibit, rather than activate, transcription. Subtle differences in the nucleotide sequences of CREs change recognition and binding affinities by different homo and heterodimers (Meyer, 1993). A specific DNA binding sequence in AEG's promoter may promote the binding of CREM and therefore decrease the transcription rate during chronic isoproterenol stimulation.

4.3.2 Epididymal AEG is not Affected by Isoproterenol

AEG expression in the epididymis is not regulated by ß-adrenergic agonists. Again, neurohormonal responsiveness is dependent to some extent on the presence of receptors. Only a few studies have examined the presence of ß-receptors in the epididymis. The innervation of the rat epididymis includes noradrenergic fibers, especially in the cauda. (see section 1.2.2) Transepithelial chloride secretion in the epididymis has been shown to be stimulated by a_1, $ß_1$

and $ß_2$ adrenoreceptor agonists, which provides evidence for the existence of $ß_1$ receptors (the subtype in parotid acinar cells) in the epididymal epithelium (Leung, 1992). In fact, stimulation of ß-adrenoreceptors in the epididymal epithelium has been shown to lead to an increase in intracellular cAMP concentrations (Wong, 1990). But the presence of ß-adrenergic receptors and cAMP does not necessarily lead to the ability to regulate gene transcription. The only known effect of increasing cAMP concentration in the epididymis is the opening of various ion exchangers and channels. The control of gene expression depends, in part, on the presence of the appropriate cAMP-dependent kinase and transcription factors in a particular cell type. No experimental investigations to date have demonstrated that these are indeed synthesized in the epididymis, which could account for the failure to effect transcription.

An alternate explanation may lie in the specific distribution of adrenergic receptors within the rat epididymis, specifically the cauda. (see section 1.2.2) The epididymal immunostaining pattern for AEG, which has shown a predominance of staining in the supranuclear region principal cells of the distal caput and corpus, the lumen distal to corpus, and in the clear cells of the cauda is consistent with AEG being synthesized in the principal cells, secreted in the lumen, and being partly reabsorbed by clear cells. (see section 1.4.2) Many secretory tissues divide the protein secretion and electrolyte/water concentration functions amoung different epithelial cell types. For example, in the parotid gland, the acinar cells synthesize the majority of the secretory proteins, while the ductal cells are responsible for water/electrolyte balance. (see section 1.3.1) It is possible that the ß-adrenergic receptors are distributed in the clear cells but not the principal cells where they could influence epididymal AEG expression. Finally, the relative concentration of each

receptor type, interaction between transcriptional factors and of DNA binding sites may give one hormone primacy over another. Isoproterenol may be unable to decrease epididymal AEG in the presence of androgens.

4.4 Integration-Tissue specific Gene Regulation

It has become increasingly apparent that eukaryotic transcription is subject to a highly complex interaction between many different factors which can exert either a positive or negative influence. The ultimate aim of understanding differential hormonal regulation of expression must be to understand how multiple positive and negative control circuits operate together to determine the level of gene expression during development and in response to the environment (Jackson, 1991). The regulation of a single gene in a tissue is achieved through the appropriate combination of HREs, silencers, tissue-specific promoter elements, and the basal promoter elements. Transcriptional response to hormones in different tissues and cells may be controlled by limiting the tissue-specific expression and concentration of various hormone receptors (Clark, 1992). Genes which are regulated through more than one hormone have been described. Neurotransmitters released by neuroendocrine cells and nerve endings have been shown to be involved along with androgen in the regulation of gene expression in the prostate gland of the rat. Rat Prostatic binding protein (PBP) expression *in vivo* is partially regulated by both ß-adrenergic and androgen receptor-mediated pathways, (Guthrie, 1990) which suggests that a single gene can posses both an ARE and CRE sequence in the promoter region.

There are many examples of single genes which are expressed in several different tissues at different levels. The mouse major urinary protein II (MUP II) gene is expressed in both the liver and the mammary gland, where it make

up 1 and 0.02% of the total RNA respectively. The other 5 MUP genes are also expressed at different levels in an assortment of tissues. The tissue-specific regulation of each MUP gene is hypothesized to be brought about by the presence of distinct tissue-specific regulatory factors (Shahan, 1987). Transferrin (Tf), an iron-binding glycoprotein, is synthesized in the liver, but is also expressed in the testis of humans and rats. In rat liver, expression of Tf in hepatocytes is constitutive, and is not influenced by steroids or iron levels. In the testis, Tf is produced by Sertoli cells in response to FSH, insulin, and retinol. These tissues arise from different embryologic origin: the hepatocytes from the endoderm, the Sertoli cells from mesoderm. *In vitro* binding assays have revealed the existance of 5 protein binding sites in the 5' promoter region of the gene. The binding of transcriptional factors to two of these sites, named proximal region (PR) I and II, are tissue-specific. In hepatocytes, the liver-enriched transcriptional factors C/EBPα and HNF-4 may interact with the PRII and PRI regions of the transferrin gene promoter, respectively. In Sertoli cells, the transcriptional factors interacting with the promoter are different, and do not cross react with antibodies to C/EBPα or HNF-4. These findings support the idea that the Tf gene requires different combinations of factors in different subsets of cells to achieve tissue-specific expression (Zakin, 1992).

Like Tf, AEG is expressed in tissues of different embryologic origin, the parotid and the epididymis, and is regulated differently at the level of transcription. The next stage of investigation will involve the sequencing of the promoter regions of the AEG gene to better understand the molecular mechanisms for tissue-specific regulation.

5.0 Appendix

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AACTCCTCAGGAAGACCAGCAGAGTCAACTAACCTGGACCCTTGGTAGCTCCCGGCGACTGAATCATTAAGCAAA  75
**************************************************************************** 75

GGGACAATATCTCATTCTGCTCTGAAATAGAACC ATG GCA TTA ATG TTA GTG CTG TTG TTC CTG  140
********************************** *** *** *** *** *** *** *** *** *** ***  140
                                    Met Ala Leu Met Leu Val Leu Leu Phe Leu   10

GCT GCT GTA TTG CCA CCA TCT CTT CTT CAA GAT ACC ACT GAT GAA TGG GAT AGA GAT 197
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 197
Ala Ala Val Leu Pro Pro Ser Leu Leu Gln Asp Thr Thr Asp Glu Trp Asp Arg Asp  29

CTT GAG AAT TTG TCA ACC ACT AAA CTG TCA GTC CAA GAA GAG ATC ATA AAC AAG CAC 254
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 254
Leu Glu Asn Leu Ser Thr Thr Lys Leu Ser Val Gln Glu Glu Ile Ile Asn Lys His  48

AAC CAA TTG AGA CGA ACG GTT TCT CCG TCT GGT AGT GAC TTA CTA AGA GTG GAA TGG 311
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 311
Asn Gln Leu Arg Arg Thr Val Ser Pro Ser Gly Ser Asp Leu Leu Arg Val Glu Trp  67

GAC CAT GAT GCT TAT GTG AAC GCT CAG AAA TGG GCA AAC AGG TGC ATT TAC AAT CAC 368
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 368
Asp His Asp Ala Tyr Val Asn Ala Gln Lys Trp Ala Asn Arg Cys Ile Tyr Asn His  67

AGT CCT CTA CAA CAC AGG ACA ACC ACA TTA AAA TGT GGT GAG AAT TTG TTC ATG GCA 425
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 425
Ser Pro Leu Gln His Arg Thr Thr Thr Leu Lys Cys Gly Glu Asn Leu Phe Met Ala 105

AAT TAC CCT GCA TCG TGG TCT TCT GTA ATC CAA GAT TGG TAT GAT GAA TCC CTT GAT 482
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 482
Asn Tyr Pro Ala Ser Trp Ser Ser Val Ile Gln Asp Trp Tyr Asp Glu Ser Leu Asp 124

TTT GTC TTT GGT TTC GGC CCA AAA AAA GTT GGT GTT AAA GTC GGA CAC TAT ACT CAG 539
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 539
Phe Val Phe Gly Phe Gly Pro Lys Lys Val Gly Val Lys Val Gly His Tyr Thr Gln 143

GTT GTT TGG AAT TCA ACT TTC CTG GTT GCA TGT GGA GTT GCT GAA TGC CCT GAC CAA 596
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 596
Val Val Trp Asn Ser Thr Phe Leu Val Ala Cys Gly Val Ala Glu Cys Pro Asp Gln 162

CCA TTG AAA TAC TTT TAT GTT TGT CAC TAT TGT CCT GGT GGC AAT TAT GTA GGA AGA 653
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 653
Pro Leu Lys Tyr Phe Tyr Val Cys His Tyr Cys Pro Gly Gly Asn Tyr Val Gly Arg 181

CTA TAC TCA CCT TAC ACA GAA GGA GAA CCT TGT GAC AGT TGT CCT GGT AAT TGT GAA 710
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 710
Leu Tyr Ser Pro Tyr Thr Glu Gly Glu Pro Cys Asp Ser Cys Pro Gly Asn Cys Glu 200
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GAT GGG CTG TGC ACC AAT AGT TGT GAA TAT GAA GAT AAT TAT TCT AAC TGT GGC GAT 767
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 767
Asp Gly Leu Cys Thr Asn Ser Cys Glu Tyr Glu Asp Asn Tyr Ser Asn Cys Gly Asp 219

CTG AAG AAG ATG GTG AGC TGC GAC GAT CCA CTT CTT AAA GAA GCT TGC AGA GCT TCA 824
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 824
Leu Lys Lys Met Val Ser Cys Asp Asp Pro Leu Leu Lys Glu Gly Cys Arg Ala Ser 238

TGC TTC TGT GAA GAC AAA ATT CAT TAA ATTTCCAGTCCACATAATCAGGACCATGTAGAAAAGGAA 890
*** *** *** *** *** *** *** *** *** **********************T**************** 890
Cys Phe Cys Glu Asp Lys Ile His End

AATACCCTCTACTTAGTCTTATCATGTCCCACCAAAAATATGTAGGTTTAGTCACTGAAATAATTCCAAATGGT  965
********************C***** ----------------------------------------------

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-------------------------------------------------------------------------

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-------------------------------------------------------------------------

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-------------------------------------------------------------------------

CTAATTATCTCCGGAGAAAACATTCACATTAATTGTCTTCTATTTCTTCTCACCATTCATTATTCTTCACATTCA 1340
-------------------------------------------------------------------------

TCAGAATTAGTGGTTTAAATTCTAAACTACCATTTATGTTTTGTTGTCGGGTCTTTAAGAATGATATTAAAATGT 1415
-------------------------------------------------------------------------

AACTTAATAAACAGAATTTGCTTGTTCAGGGGTAATGACCTTGGTTGCTTCAGAAAAAAAA-TAAATCTTAATCT 1489
-----------------------------------------**********************A*************  952

TAGCATATTAAAAAAAAAAAAAA------
C****----***************AAAAAA
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DNA And Deduced Amino Acid Sequence of AEG cDNA

The top line is the nucleotide sequence of the 1500-bp cDNA; the middle line is the nucleotide sequence of the 950-bp cDNA; and the bottom line is the predicted amino acid sequence of both clones. Identical bases are represented by (*), while bases not present in a clone are indicated by (-). The consensus inititation sequence, ACCATGG, and the polyadenylation signal, AATAAA, are underlined. (From Charest, 1988)

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